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(54) Title: INTERCALATORS HAVING AFFINITY FOR DNA AND METHODS OF USE (57) Abstract Intercalator compounds are provided which are comprised of intercalator moieties, or substituted intercalator moieties, having one or more functionalized chains, or moieties, and which compounds provide a high affinity for binding to the DNA molecule and show reduced self-quenching, while providing superior transport kinetics. The compounds have been found to provide enhanced fluorescence when bound to a DNA molecule within a fluorescent flow cytometry environment which is about eight to ten times brighter in fluorescence than "bis" structure conventional intercalating agents and other known intercalating agents utilized in flow cytometry environment.		

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INTERCALATORS HAVING AFFINITY FOR DNA AND METHODS OF USE

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BACKGROUND

The present invention relates to intercalator compounds, and the use of such compounds, each of which is comprised of an intercalator moiety, or a substituted intercalator moiety, derivatized with one or more functionalized chains, or moieties, and which compounds have high affinity for binding to a DNA molecule. These intercalator compounds exhibit improved binding to a DNA molecule within known methodologies requiring intercalator insertion into the DNA molecule. Still, the invention relates to enhanced binding of DNA molecules by an intercalator-functioning segment utilized in labeling, capture, therapeutic insertion, assay and the like, with improved performance of the intercalator due to the increased utilization efficiency of the compounds.

The term "intercalator" was introduced into the chemistry field over 30 years ago to describe the insertion of planar aromatic or heteroaromatic compounds between adjacent base pairs of double stranded DNA (dsDNA). Many DNA intercalating compounds elicit biologically interesting properties. It is generally agreed that these properties are related to their reactivity with DNA. In the search for more active compounds, it is logical to design molecules with the highest possible affinity for DNA. In 1990, it was reported that complexes of ethidium homodimer with dsDNA performed at ratios of one dimer per four to five base pairs, and were stable to electrophoresis on agarose gels. This allowed fluorescence, detection and quantitation of DNA fragments with picogram sensitivity after separation and complete absence of background stain. Such a result has been sought through various manipulations of intercalator compounds, for example, by functional compounds made up of

DNA intercalating dyes. As a result of these efforts, DNA intercalating agents utilizing ethidium bromide have been used in various DNA analytical procedures.

Various reported DNA intercalating agents utilizing ethidium bromide have been used in a multitude of DNA analytical procedures, for example:

Christen, et al., "An Ethidium Bromide-Agarose Plate Assay for the Nonradioactive Detection of cDNA Synthesis", Anal. Biochem., 178 (2), May 1, 1989, pp. 269-272, report ethidium bromide was used to determine the success of cDNA synthesis reactions. Since ethidium bromide in agarose can be used to quantitate RNA and DNA, conditions under which the greater fluorescence of double-stranded DNA is utilized were devised to assay double stranded DNA synthesis from mRNA. Ethidium bromide at 5 micrograms/ml in agarose allowed quantitative detection of cDNA in the range of 0.03 to 0.0015 microgram. Sodium dodecyl sulfate had an adverse effect on the measurement of cDNA. Subsequent cDNA analysis by alkaline gel electrophoresis and staining in 5 micrograms/ml ethidium bromide allowed accurate and rapid sizing of cDNA and required only 0.01-0.05 microgram cDNA.

Petersen, S.E., "Accuracy and Reliability of Flow Cytometry DNA Analysis Using a Simple, One-Step Ethidium Bromide Staining Protocol", Cytometry, 7 (4), July, 1986, pp. 301-306, reports that sources of variation and error were investigated for a simple flow cytometric analysis of DNA content of detergent-isolated nuclei stained with ethidium bromide.

In "Ethidium Bromide in the Detection of Antibodies to DNA and of Circulating DNA by Two-Stage Counterimmunoelectrophoresis", J. Immunol. Methods, 85 (1), Dec. 17, 1985, pp. 217-220, Riboldi, et al., report that in an attempt to overcome the limitations of counterimmunoelectrophoresis in the detection of precipitating anti-DNA antibodies or circulating DNA, ethidium bromide was used to increase the visibility of the precipitating lines and to confirm their specificity.

W.A. Denny reported in "DNA-Intercalating Ligands as Anti-Cancer Drugs: Prospects for Future Design", Anticancer Drug Des., 4 (4), Dec., 1989, pp. 241-263, that interest in DNA-intercalating ligands as anti-cancer drugs has developed greatly since the clinical success of doxorubicin.

5 A number of agents have been described for labeling nucleic acids, whether probe or target, for facilitating detection of target nucleic acid. Suitable labels may provide signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption, magnetism or enzymatic activity, and include, for example, fluorophores, chromophores, radioactive isotopes,
10 enzymes, and ligands having specific binding partners.

 Fluorescent dyes are suitable for detecting nucleic acids. For example, ethidium bromide is an intercalating agent that displays increased fluorescence when bound to double stranded DNA rather than when in free solution. Ethidium bromide can be used to detect both single and double stranded nucleic
15 acids, although the affinity of ethidium bromide for single stranded nucleic acid is relatively low. Ethidium bromide is routinely used to detect nucleic acids following gel electrophoresis. Following size fractionation on an approximate gel matrix, for example, agarose or acrylamide, the gel is soaked in a dilute solution of ethidium bromide.

20 The use of fluorescence labeled polynucleotide probes and polynucleotide hybridization assays have been reported. According to these methods, probes are prepared by attaching a particular absorber-emitter moieties to the three prime and five prime ends of the nucleic acid fragments. The fragments are capable of hybridizing to adjacent positions of a target DNA
25 so that if both fragments are hybridized, the proximity of the absorber and emitter moieties results in the detectable emitter fluorescence. According to these methods, the fluorescent dye is introduced into the target DNA after all in vitro nucleic acid polymerizations have been completed. The inhibitory effects of intercalating agents on nucleic acid polymerases have been described
30 in numerous locations.

DNA binding dyes are useful as antibiotics because of the inhibitory effects of nucleic acid replication processes that result from the agent binding to the template. The use of intercalating agents for blocking infectivity of influenza or herpes viruses have been reported. It has also been reported and described that a number of DNA binding agents, both intercalators and nonintercalators, inhibit nucleic acid replication. For example, ethidium bromide inhibits DNA replication.

Methods have been provided for detecting a target nucleic acid in a sample. These methods comprise the steps of (a) providing an amplified reaction mixture that comprises a sample, a DNA binding agent, where said agent is characterized by providing a detectable signal when bound to double stranded nucleic acid, which signal is distinguishable from the signal provided by said agent when it is unbound, and reagents for amplification; (b) determining the amount of signal produced by the mixture of step (a); (c) treating said mixture under conditions for amplifying the target nucleic acid; (d) determining the amount of said signal produced by the mixture of step (c); and (e) determining if amplification has occurred. These DNA binding intercalating agents, such as ethidium bromide or ethidium homodimer allow fluorometric study of the interaction of various molecules with DNA.

The intercalating agent useful for DNA binding or detecting amplified nucleic acids is an agent or moiety capable of insertion between stacked base pairs in the nucleic acid double helix. Intercalating agents such as ethidium homodimer and ethidium bromide fluoresce more intensely when intercalated into double stranded DNA than when bound to single stranded DNA, RNA, or in solution. Other uses of intercalators have been in the field of separation and isolation or purification of nucleic acids from complex biological or clinical specimens.

Various methods of separating deoxyribonucleic acids (DNA) from liquid biological samples are known in the art, but are very time consuming or otherwise plagued by complication. It is known that DNA adheres to

nitrocellulose. The liquid sample containing DNA is applied to a nitrocellulose filter and the DNA adheres or binds to the filter.

Another method of separating DNA from samples is ultracentrifugation with sucrose or cesium chloride density gradients. The DNA is separated from other macromolecules such as proteins by this method according to the buoyant density or sedimentation coefficient. The biological sample is layered onto the density gradient in a centrifuge tube and is spun at very high speeds for long periods of time for DNA to travel through the density gradient. This method, although satisfactory, is very time consuming and labor intensive. The centrifugation time may be 20 hours or more per sample. Furthermore, if the sample is spun too long, the DNA will not only separate from the sample but also will pass entirely through the gradient to the very bottom of the centrifuge tube along with other constituents in the sample. Therefore, this method is also not suitable as a fast and easy method for separating DNA from complex samples.

Agarose polyacrylamide gel electrophoresis is also used to separate DNA from biological samples. In this method, the sample is applied to one end of a glass or plastic receptacle containing the gel and an electric current is applied across the length of the receptacle. The negatively charged nucleic acid molecules move toward the anode, the larger molecules moving more slowly. The rates of migration of the molecules depend on their molecular weights and on the concentration and degree of cross linking in the gel material. The DNA is then removed from the gel by cutting out that portion of the gel in which the DNA is located and finally extracting the DNA. Again, this method is time consuming and labor intensive, and the DNA must still be separated from the gel. When DNA is separated by the electrophoresis gel method or by centrifugation, it is necessary for the DNA to be stained in some manner to be visualized. Typically, ethidium bromide (EtBr) has been used as the staining agent. Ethidium bromide adheres to the DNA by intercalation between the base pairs of the double helix structure of the DNA.

More recently, an ethidium homodimer has been synthesized and introduced with bifunctional intercalators in order to allow fluorometric study including the interaction of such molecules with DNA. It has been determined that the ethidium homodimer ("EthD") binds to double stranded DNA ("dsDNA") about two (2) orders of magnitude more strongly than ethidium bromide. Complexes of EthD with dsDNA have performed at a ratio of one dimer per 4 to 5 base pairs and were found to be stable to electrophoresis on agarose base. On binding to dsDNA, the fluorescence quantum yield of the dimer increases 40 fold independent of nucleotide sequence.

Stable dsDNA-fluoropore complexes can be formed to obtain anywhere from several to several thousand fluoropores each, as desired. Under suitable controlled conditions these complexes do not transfer dye to other nucleic acids or proteins. An important property of these complexes is that their fluorescence emission intensity is a linear function of the number of intercalated dye molecules. As high sensitivity fluorescence detection apparatus becomes more generally available, the ability to use dyes to replace, for example, radioactivity for sensitivity detection of DNA, is becoming more and more valuable.

Dye dsDNA complexes represent a novel family of fluorescence labels with a wide range of spectroscopic properties whose composition, structure and size can be tailored to particular applications. DNA molecules can be readily derivatized to attach biotin, digoxigenin or any number of other substituents that can be recognized by avidin or antibodies. Such derivatized DNA molecules loaded with dye may allow detection at much higher sensitivity in numerous applications, for example, immunoassay, fluorescence, and in situ hybridization of chromosomes and the like that currently use other fluorescence labels.

Probes with a double stranded region, which provide intercalation sites and a single stranded region to allow recognition by hybridization of specific target sequences, offer another approach to the generation of versatile

fluorescent labels. Development of conditions that allow clear discrimination between the binding of intercalators to single and double stranded nucleic acids is an essential prerequisite to the use of such probes.

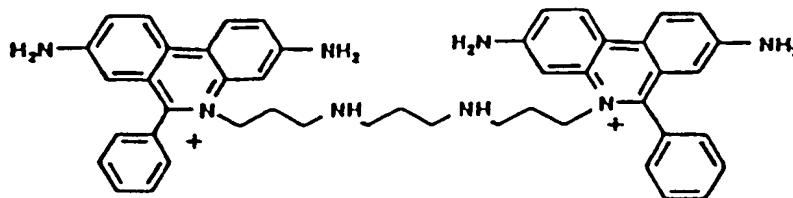
Fluorescent probes are valuable reagents for the analysis and separation of molecules and cells. Some specific examples of their application are identification and separation from a subpopulation of cells in a mixture of cells by the techniques of fluorescence, flow cytometry, fluorescence-activated cell sorting, and fluorescence microscopy. Other applications include determination of a concentration of a substance or member of a specific binding pair that binds to a second species, or member of the specific binding pair, e.g., antigen-antibody reactions in an immunofluorescent assay. Still another application is the localization of substance in gels and other insoluble supports by the techniques of fluorescence staining.

Choice of fluorescers for these purposes is hampered by various constraints; one being the absorption and emission characteristics of the fluorescer since many ligands, receptors and other binding pair members, as well as other extraneous materials associated with the sample, for example, blood, urine and cerebrospinal fluid, will auto-fluoresce and interfere with an accurate determination or quantification of the fluorescent signal generated by the fluorescent label when the sample is exposed to the appropriate stimulus. Another consideration is the quantum efficiency of the fluorescer. Yet another concern is self-quenching; this can occur when the fluorescent molecules interact with each other when in close proximity. An additional concern is the non-specific binding of the fluorescer to other compounds or even with the test container.

It has been shown that dsDNA forms highly fluorescent complexes with the bis-intercalator EthD. Observations regarding the bis-intercalator EthD suggest that the intercalator can be exploited to generate a family of highly fluorescent stable dsDNA-dye complexes with distinctive properties. Such complexes could be exploited by multiplex detection of dsDNA fragments, as

well as many analytical applications in which appropriately diversified dsDNA fragments labeled noncovalently with different dyes could be used as a unique family of fluorescent probes:

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However, this compound may have a tendency to self-quench when bound to DNA.

In flow cytometry apparatuses, cells or other particles are caused to flow in a liquid flow stream so as to facilitate the investigation of certain characteristics thereof. In general, a flow cytometry apparatus is useful for identifying the presence of certain cells or particles of interest, enumerating those cells or particles and, in some instances, providing a sorting capability so as to be able to collect those cells or particles of interest. In a typical flow cytometry apparatus, a fluid sample containing cells is directed through the apparatus in a rapidly moving liquid stream so that each cell passes serially, and substantially one at a time, through a sensing region. Cell volume may be determined by changes in electrical impedance as each cell passes through the sensing region. Similarly, if an incident beam of light is directed at the sensing region, the passing cells scatter such light as they pass therethrough. This scattered light has served as a function of cell shape and size, index of refraction, opacity, granularity, roughness and the like. Further, fluorescence emitted by labeled cells, or autofluorescent cells, which have been excited as a result of passing through the excitation energy of the incident light beam is detectable for identification of cells having fluorescent properties. After cell analysis is performed by the flow cytometry apparatus, those cells that have

been identified as having the desired properties may be sorted if the apparatus has been designed with such capability.

Instruments such as flow cytometry apparatuses are particularly useful for researchers and investigators studying various responses, reactions and functions of the immune system. Immunofluorescence studies, as well as fluorescence immunoassays, assist the investigator in identifying and targeting select cells of interest so that disease states, conditions and the like may be properly characterized. In addition to immune system investigations, fluorescence analysis is also quite beneficial in cell biology and morphology investigations, including the study of the substrate of cellular material.

In relying upon fluorescence to provide data and information about cells, the mechanics of performing tests for the fluorescence response is a major consideration in the design of the instrument as well as the results obtained. Specifically, the fluorescent markers, whether such markers be fluorescent stains or dyes, are typically excited by light energy. Usually there is an optimal wavelength which provides the greatest level of excitation for the fluorochromatic marker being used. Once excited, fluorescence emission occurs typically at wavelengths different from the wavelength of excitation. Fluorescence analysis instruments, whether fluorescence microscopes, image analyzers or flow cytometers, are generally designed to detect the fluorescence emission at the wavelength of emission maxima where the fluorescence signal is strongest.

Before the discovery and publication of the utilities of ethidium homodimer as an important intercalator, the usual intercalator of choice was ethidium bromide. Uses of the ethidium bromide intercalators include fluorometric methodologies, quantitative fluorescences of DNA intercalated ethidium bromide on agarose gels, ethidium bromide-agarose plate assay or detection of false DNA analysis and the like. Ethidium bromide and propidium bromide were further used in flow cytometry, as well as applications for direct electronic imaging, direct and rapid quantitation of fluorescence and

electrophoretic gels in application as ethidium bromide-stain DNA. Ethidium bromide has also been used to increase the visibility of the precipitant lines and to confirm the specificity in two stage counter immunoelectrophoresis methodologies for detection of participating anti-DNA antibodies or circulating DNA. Utilization of ethidium bromide as an intercalator in numerous environments, as well as the more recent utilization of the ethidium homodimer intercalator are well documented in the literature and present the leading edge of intercalator methodology and efficiency.

In a somewhat different application of ethidium bromide as a staining agent, ethidium bromide has been linked to a solid support. U.S. Patent No. 4,119,521, issued to Chirikjian on Oct. 10, 1978, discloses a fluorescent DNA intercalating agent derivative of activated polysaccharides. The derivatives in the patent function as fluorescent stains to provide direct visualization of the DNA and their fractions, under the excitation of shortwave, ultraviolet radiation. The intercalating agents used in the patent are ethidium halides, with the preferred agent being ethidium bromide. This agent is coupled covalently to an activated polysaccharide such as agarose.

Utilization of ethidium bromide as an intercalator for use in numerous environments, as well as the more recent utilization of the ethidium homodimer intercalator are well documented in the literature and present the leading edge of intercalator methodology and efficiency. However, there remains an ever present need to improve utilization of the intercalators and viability of the use of intercalators with DNA, specifically addressing (1) high affinity for binding the intercalators to the DNA molecule; (2) reduction of self-quenching; and (3) providing superior transport kinetics. Intercalators possessing these qualities reduce the amount of intercalator required for performing one of the many functions involved in the aforementioned methodologies which can also enhance methodologies. In addition, improvement in accuracy and reliability of the various uses of interest is of continuing concern.

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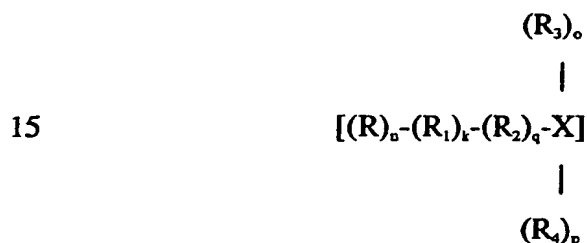
SUMMARY

Broadly, the invention provides a compound having an "I" moiety bonded to one or more "T" moiety. The general formula of the compounds are represented by:

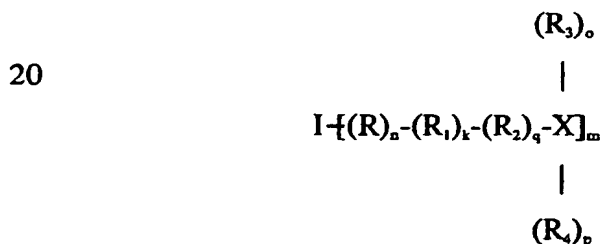


wherein the I moiety denotes an aromatic or heteroaromatic segment; the T moiety denotes a "tail" or "chain" moiety; and m is an integer from 1 to 5. When m is more than 1, T can be similar or different from one another.

10 In one aspect, the present invention provides a compound having an I moiety bonded to one or more T moiety, the T moieties, when more than one T moiety are present, are the same or different from one another, the T moiety having the formula of:



and the compound having a formula:



25 wherein: I is an aromatic or heteroaromatic segment;
X is a heteroatom selected from the group consisting of nitrogen and sulfur;
R, R₁ and R₂ are the same or different from one another and are alkyl, alicyclic, heteroalicyclic, aromatic or heteroaromatic groups;

R_3 and R_4 are hydrogen when X is nitrogen; R_3 and R_4 are methyl, ethyl, or phenyl groups when X is sulfur;

k is zero or an integer from 1 to 10;

q is zero or an integer from 1 to 10;

5 n is an integer from 20 to 20;

m is an integer from 1 to 5;

o is zero or one; and

p is zero or one;

and the acid addition salts thereof;

10 provided that the I moiety is not phenantridium when: X is nitrogen; R_3 and R_4 are hydrogens R_1 and R_2 are methylene groups; o is 1; p is 1; m is 1; and the acid addition salts thereof;

provided that the I moiety is not phenantridium when: X is nitrogen; R_3 and R_4 are hydrogens; R_1 and R_2 are methylene groups; o is 1; p is 1; m is 2; a first T moiety directly bonded to the I moiety in which n is 2, k is 1 and q is zero; a second T moiety, directly bonded to the first T moiety, in which n is 2, and k and q are zero; and acid addition salts thereof;

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provided that the I moiety is not phenantridium when: X is nitrogen; R_3 and R_4 are hydrogens; R_1 and R_2 are methylene groups; o is 1; p is 1; m is 2; a first T moiety directly bonded to said I moiety in which n is 2, k is 1 and q is zero; a second T moiety, directly bonded to the first T moiety, in which n is 2, k is 1 and q is 1 and acid addition salts thereof; and

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provided that the I moiety is not phenantridium when: X is nitrogen; R_3 and R_4 are hydrogen; o is 1; p is 1; m is 3; n is 2; k is 1; q is zero; and acid addition salts thereof.

25

The invention provides compounds comprised of intercalator moieties or substituted intercalator moieties having a functionalized chain, which compounds provide a high affinity for binding to the DNA molecule and show reduced self-quenching while providing superior transport kinetics. The inventive intercalators have been found to provide enhanced fluorescence when

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bound to a DNA molecule within a fluorescent flow cytometry environment which is about eight to ten times brighter in fluorescence than ethidium homodimer utilized in the same flow cytometry environment. Because of the enhancement of fluorescence, the detection of DNA hybridization can be accomplished using much lower concentrations of intercalator compounds of the present invention than using conventional intercalating agents, such as ethidium homodimer or ethidium bromide. Using the same concentrations, intercalator compounds of the present invention can detect far less amounts of DNA hybridization than can conventional intercalating agents. Thus, the intercalator compounds of the present invention are far more sensitive than the known intercalating agents in detecting DNA hybridization.

Improvements in flow cytometry, fluorescence in-situ hybridization assays, gel electrophoresis, DNA detection, immunoassay for DNA, and other DNA studies are substantial. The use of intercalators with DNA and multiple methods have shown that the ethidium homodimer is about two orders of magnitude brighter than conventional staining methodology, i.e., ethidium bromide. However, the intercalator compounds in accordance with the present invention provide, for example, a dye which exhibits an eight to ten-fold increase in brightness over that of EthD in the same environment, or about a thousandfold improvement over more conventional staining methodologies. With pre-staining and post-electrophoresis detection, the sensitivity level of radioimmunoassay detection of DNA is now attainable with fluorophores. The compound compositions provided by this invention extends the limits of detection by up to tenfold over EthD, thereby providing a potential for new uses in applications of intercalators for the study of DNA analysis, as well as therapeutics and the like.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a FACScan™ display for side scatter versus forward scatter;

FIGURE 2 is a histogram of fluorescence intensity (abscissa) versus frequency of events (ordinate);

FIGURE 3 is a scattergram representation for side scatter (SSC on abscissa) versus fluorescence intensity (ordinate);

FIGURE 4 is a FACScan™ display for side scatter versus forward scatter;

FIGURE 5 is a histogram of fluorescence intensity (abscissa) versus frequency of events (ordinate);

FIGURE 6 is a scattergram representation for side scatter (SSC on abscissa) versus fluorescence intensity (ordinate);

FIGURE 7 is a photographic representation of a UV light irradiated agarose electrophoresis gel performed on enzyme BAMH nicked PBR322 plasmid DNA;

FIGURE 8 is a hybridization saturation plot for equivalents;

FIGURE 9 shows hybridization titration curves for fluorescence intensity versus equivalents;

FIGURE 10A is a FACScan™ display (SSC vs FSC) of a typical distribution of lysed white cells;

FIGURE 10B is a FACScan™ display of the same blood sample as in FIGURE 10A with unstained chicken erythrocyte nuclei ("CEN") added;

FIGURE 10C is a FACScan™ display of the same sample lysed with white blood cell diluent ("WBC DIL");

FIGURE 10D is a FACScan™ display of the same sample as presented in FIGURE 10C, but with CEN;

FIGURE 10E is a FACScan™ display of the same sample lysed in the same diluent as FIGURE 10D, but with 0.5 µg/ml of nucleated red blood cell ("NRBC") dye;

FIGURE 10F is a FACScan™ display of the same sample lysed in the same diluent as FIGURE 10D, but with 0.25 μ g/ml of NRBC dye;

FIGURE 11 is a graphical representation of the efficiency of ³²P radiolabelled, restriction enzyme-nicked plasmid DNA capture onto phenathridinium activated polystyrene microparticles prepared as described in Example 6;

FIGURE 12 is a graphical representation of the efficiency of ³²P radiolabelled plasmid DNA capture onto phenathridinium activated carboxymethyl sepharose beads synthesized as described in Example 6;

FIGURES 13A, 13B, and 13C show the structures or representative I moieties;

FIGURE 14 is a reaction scheme for the synthesis of compound 24 and its precursors;

FIGURE 15 is a schematic representation of intercalator derivatized solid phase microparticle;

FIGURE 16 shows the structural formula of compounds 25, 26 and 27;

FIGURE 17A, 17B, and 17C show a reaction scheme for the synthesis of compounds 28a-42a;

FIGURE 18 is a reaction scheme for the synthesis of compound 50 and its precursors;

FIGURE 19 is a reaction scheme for the synthesis of compounds 55, 55a, and their precursors;

FIGURE 20 shows the structural formula of compounds 55a-55p;

FIGURE 21 is a reaction scheme for the synthesis of compounds 58, 58a, and their precursors;

FIGURE 22 shows the structural formula of compounds 58a-58p;

FIGURE 23 is a reaction scheme for the synthesis of compound 64 and its precursors;

FIGURE 24 is a reaction scheme for the synthesis of compounds 68, 68a, and their precursors;

FIGURE 25 shows the structural formula of compounds 68a-68p;

FIGURE 26 is a reaction scheme for the synthesis of compounds 71, 71a, and their precursors;

FIGURE 27 shows the structural formula of compounds 71a-71p;

FIGURE 28 is a reaction scheme for the synthesis of compound 80 and
5 its precursors;

FIGURE 29 is a reaction scheme for the synthesis of compound 85 and its precursors;

FIGURE 30 is a reaction scheme for the synthesis of compound 90 and its precursors;

10 FIGURE 31 is a comparison of relative fluorescence intensities obtained from staining DNA by compound 24, ethidium bromide, propidium iodide, and ethidium homodimer; and

FIGURE 32, is a comparison of relative fluorescence intensities obtained from staining DNA by compounds 24, 25, 26, 27, and ethidium
15 bromide.

DETAILED DESCRIPTION

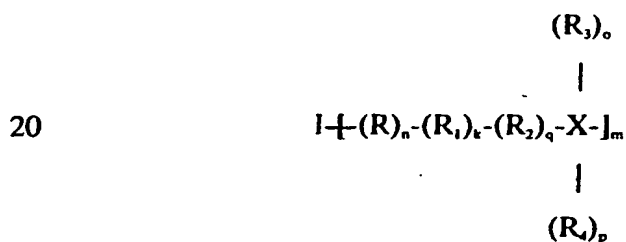
Broadly, the present invention provides an intercalator having an I moiety bonded to one or more T moiety. The general formula of the compounds are represented by:



wherein the I moiety denotes an aromatic or heteroaromatic segment; the T moiety denotes a "tail" or "chain" moiety; and m is an integer from 1 to 5, preferably from 1 to 3.

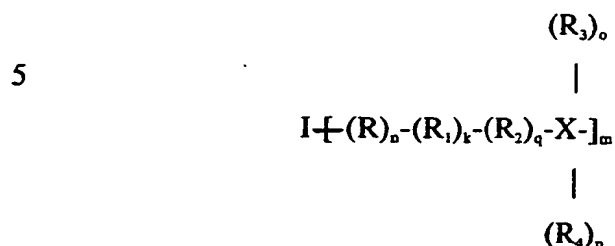
When the I moiety contains a mono-quaternary ammonium functionality, it is accompanied by a monovalent counter anion ("A⁻"). Examples of monovalent counter anion include chloride, bromide, iodide, hydroxide, and hydrogen phosphate.

Improved binding to the DNA molecule of intercalators and substituted intercalators is achieved which exhibit high affinity for binding, reduced self-quenching, and superior transport kinetics, especially when compared to ethidium homodimer or other bis-intercalators, by providing intercalator segments with functionalized chains forming compounds having the formula:



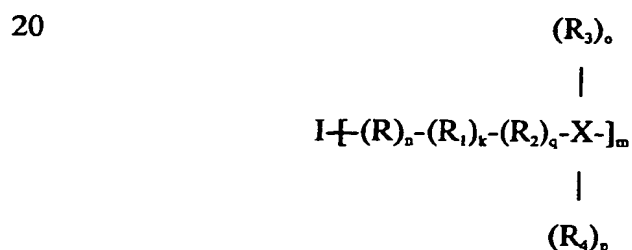
wherein I is an aromatic or heteroaromatic segment; X is a nitrogen or a sulfur; R, R₁ and R₂ are alkyl, alicyclic, heteroalicyclic, aromatic or heteroaromatic groups; R₃ and R₄ are hydrogens when X is nitrogen or methyl, ethyl or phenyl groups when X is phosphorus or sulfur; k is zero or an integer from 1 to 10; q is zero or an integer from 1 to 10; m is an integer from 1 to 5; n is an integer from 2 to 20; o is zero or one; and p is zero or one.

A compound comprised of an intercalator functionalized with chains containing heteroatoms and aliphatic, alicyclic, cyclohexyl, aromatic segments or combinations thereof having the formula:



10 wherein I is an intercalator segment; X is a main group element phosphorus or sulfur yielding, respectively polyphosphonium or polysulfonium moieties; R, R₁ and R₂ are alkyl, alicyclic, heteroalicyclic, aromatic or heteroaromatic groups; R₃ and R₄ are methyl, ethyl or phenyl groups when X is phosphorus or sulfur; k is zero or an integer from 1 to 10; q is zero or an integer from 1 to
 15 10; m is an integer from 1 to 5; n is an integer from 2 to 20; o is zero or one; and p is zero or one; where X is sulfur, o or p are zero; and where X is phosphorus, o and p are one.

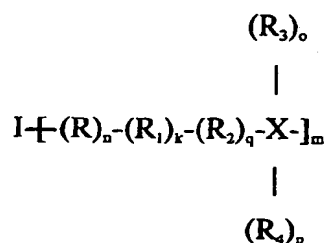
In another embodiment, it is provided a compound having the general structural formula of:



25 wherein I is an aromatic or heteroaromatic segment; X is a nitrogen or a sulfur; R, R₁ and R₂ are the same or different and are alkyl of one to four carbons, alicyclic of five to six carbons, heteroalicyclic of three to five carbons and one or two heteroatom of nitrogen, oxygen or sulfur, aromatic group of benzene,
 30 phenyl or naphthyl or heteroaromatic group of one to five carbons and one to

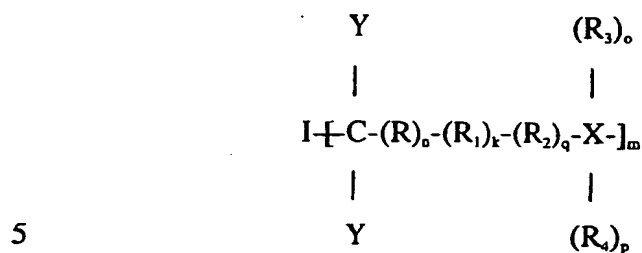
four heteroatom of nitrogen, oxygen, or sulfur; R_3 and R_4 are hydrogens when
 X is nitrogen or methyl, ethyl or phenyl groups when X is sulfur; k is zero or
 an integer from 1 to 10; q is zero or an integer from 1 to 10; m is an integer
 from 1 to 5; n is an integer from 2 to 20; o is zero or one; and p is zero or
 one.

In yet another aspect, there is provided a compound comprised of an
 intercalator functionalized with chains containing heteroatoms and aliphatic,
 alicyclic, cyclohexyl, aromatic segments or combinations thereof having the
 formula:



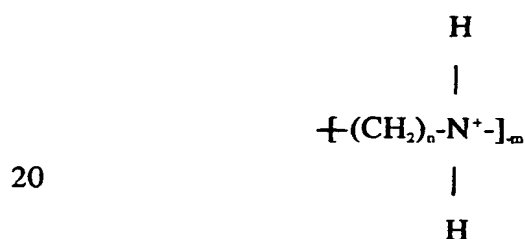
wherein I is an aromatic or heteroaromatic segment; X is a main group sulfur
 yielding polysulfonium moieties; R, R_1 and R_2 are the same or different and are
 alkyl of one to four carbons, alicyclic of five to six carbons, heteroalicyclic of
 three to five carbons and one or two heteroatom of nitrogen, oxygen or sulfur,
 aromatic group of benzene, phenyl or naphthyl or heteroaromatic group of one
 to five carbons and one to four heteroatom of nitrogen, oxygen, or sulfur; R_3
 and R_4 are hydrogens when X is nitrogen or methyl, ethyl or phenyl groups
 when X is sulfur; k is zero or an integer from 1 to 10; q is zero or an integer
 from 1 to 10; m is an integer from 1 to 5; n is an integer from 2 to 20; o is
 zero; and p is zero.

A compound comprised of aromatic or heteroaromatic segments
 functionalized with positively charged chains having the formula:

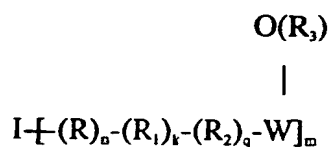


wherein Y is a side chain comprised of positively charged heteroatoms or metal
 ions in an aliphatic, alicyclic, heteroalicyclic, aromatic or heteroaromatic group
 or combinations thereof; I is an aromatic or heteroaromatic segment; X is a
 10 heteroatom; R, R₁ and R₂ are alkyl, alicyclic, heteroalicyclic, aromatic or
 heteroaromatic group; R₃ and R₄ are hydrogens when X is nitrogen or methyl,
 ethyl or phenyl groups when X is phosphorus or sulfur; k is zero or an integer
 from 1 to 10; q is zero or an integer from 1 to 10; m is an integer from 1 to
 5; n is an integer from 2 to 20; o is zero or one; p is zero or one; where X is
 15 sulfur, o and p are zero; and where X is phosphorus o and p are one.

A compound as above wherein Y has the formula:

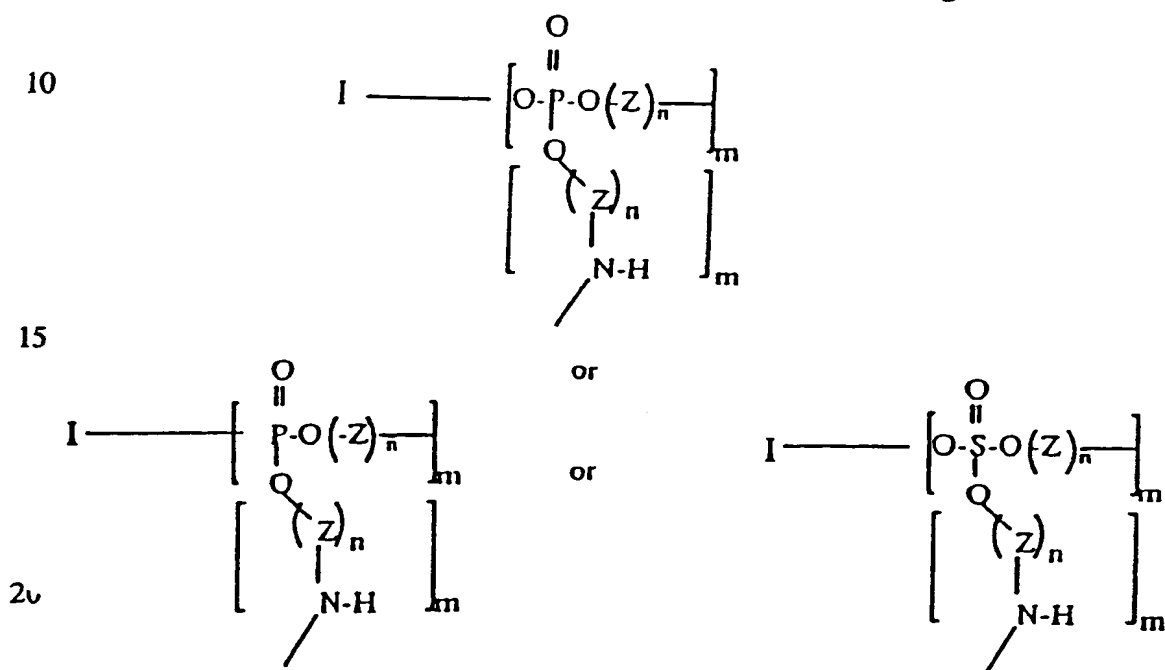


Further, a compound comprised of intercalators functionalized with
 chains containing metal atoms and alkyl, alicyclic, or aromatic segments or
 25 combinations having the formula:



wherein W is aluminum, boron or a Lewis acid metal; I is an intercalator segment; R, R₁, R₂ and R₃ are alkyl, alicyclic, or aromatic groups; k is zero or an integer from 1 to 10; q is zero or an integer from 1 to 10; m is an integer from 1 to 5; and n is an integer from 2 to 20.

- 5 An intercalator composition functionalized with positively charged chains where the positive charges are located on an aliphatic, alicyclic, aromatic or the combination thereof with a polyaminic ester group of main chain polyphosphate, polyphosphonate or polysulfate, having the formula:



- wherein I is an aromatic or heteroaromatic segment; P is a phosphorus atom; S is a sulfur atom; Z is an aliphatic, alicyclic or aromatic chain or the combination thereof; n is from 2 to 20, preferably 2 or 3; and m is from 1 to 5, preferably, 3-10.
- 25

Broadly, the present invention relates to a compound comprised of an intercalator moiety, or a substituted intercalator moiety, derivatized with functionalized chains, and the compound has high affinity for binding to a DNA molecule. In one aspect, the invention relates to use of these intercalator compounds which exhibit improved binding to a DNA molecule within known

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methodologies requiring intercalator insertion into the DNA molecule. Still, the invention relates to enhanced binding of DNA molecules by an intercalator functioning segment utilized in labeling, capture, therapeutic insertion, assay and the like, with improved performance of the intercalator due to the increased utilization efficiency of the compounds. Improved binding to the DNA molecule of intercalators and substituted intercalators is achieved which exhibit high affinity for binding, reduced self-quenching, and superior transport kinetics, especially when compared to ethidium homodimer or other bis-intercalators.

The various embodiments of the present invention inclusive of synthesis of the compounds and utilization of said compounds are shown in FIGURES 1-9, 10A-10F, 11-30. The information shown in these figures clearly demonstrates high affinity for binding, reduced self-quenching, and superior transport kinetics, especially when compared to ethidium homodimer or other bis-intercalators.

FIGURE 1 is a FACScan™ display for side scatter versus forward scatter (SSC on abscissa axis and FSC on the ordinate axis) for a typical distribution of white cells lysed with WBC DIL diluent with NRBC dye phenathridinium triamine (PTA) 24 and CEN. The quadrant thresholds were set to preclude the lymphocytes gated on the SSC versus FSC dot plot.

FIGURE 2 is a histogram of fluorescence intensity (abscissa) versus frequency of events (ordinate) for the populations of stained and unstained cells in the presence of phenathridinium triamine (PTA) 24.

FIGURE 3 is a scattergram representation for side scatter (SSC on abscissa) versus fluorescence intensity (ordinate) showing the separation of cells stained with PTA 24 (upper left hand corner, NW quadrant) from unstained cells (remainder) by fluorescence intensity.

FIGURE 4 is a FACScan™ display for side scatter versus forward scatter (SSC on abscissa axis and FSC on the ordinate axis) for a typical distribution of white cells lysed with WBC DIL diluent with NRBC dye

ethidium homodimer and CEN. The quadrant thresholds were set to preclude the lymphocytes gated on the SSC versus FSC dot plot.

FIGURE 5 is a histogram of fluorescence intensity (abscissa) versus frequency of events (ordinate) for the populations of stained and unstained cells in the presence of ethidium homodimer.

FIGURE 6 is a scattergram representation for side scatter (SSC on abscissa) versus fluorescence intensity (ordinate) showing the separation of cells stained with ethidium homodimer (upper left hand corner, NW quadrant) from unstained cells (remainder) by fluorescence intensity.

FIGURE 7 is a photographic representation of a UV light irradiated agarose electrophoresis gel performed on BAMH nicked PBR322 plasmid DNA. Loadings of 5 μ l of stock solutions were made for lanes 3-14. The following stock solutions of DNA and intercalator were used to load lanes 1-14. Lane 1: marker; Lane 2: blank; Lane 3: 20 ng/ml plasmid stained with ethidium bromide; Lane 5: 160 pg/ml plasmid stained with ethidium bromide; Lane 6: 40 pg/ml plasmid stained with ethidium bromide; Lane 7: 20 ng/ml plasmid stained with ethidium homodimer; Lane 8: 800 pg/ml plasmid stained with ethidium homodimer; Lane 9: 160 pg/ml plasmid stained with ethidium homodimer; Lane 10: 40 pg/ml plasmid stained with ethidium homodimer; Lane 11: 20 ng/ml plasmid stained with PTA 24; Lane 12: 800 pg/ml plasmid stained with PTA 24; Lane 13: 160 pg/ml plasmid stained with PTA 24; Lane 14: 40 pg/ml plasmid stained with PTA 24. In all cases, the dye/base pair ratio was 1/20. In all cases, the dye was pre-incubated with the DNA and no post-gel-electrophoresis staining was done.

FIGURE 8 is a hybridization saturation plot for equivalents of d(pT)9 added to d(pA)9 at 6.6 micromolar DNA and 3.08 micromolar dye for ethidium homodimer and PTA 24 at a dye base pair ratio of 1:4. Graphical representation of equivalents of complementary oligonucleotide d(pT)9 on the abscissa added to d(pA)9 versus relative fluorescence intensity on the ordinate generated by using the protocol described in Example 2 to compare ethidium

homodimer and PTA 24. The concentration of fluorophore in both cases was 3.08 micromolar using a two-fold statistical correction for the 2.0 molar equivalents of phenanthridinium moiety per each mole of ethidium homodimer. Both curves are normalized to background for relative fluorescence. Excitation was at 488 nm (534 nm is the excitation maximum) for this experiment and emission was at 625 nm.

FIGURE 9 shows hybridization titration curves for fluorescence intensity versus equivalents of d(pT)9 added to d(pA)9 at 6.6 micromolar DNA and 3.08 micromolar ethidium bromide and PTA 24. Graphical representation of equivalents of complementary oligonucleotide d(pT)9 abscissa versus relative fluorescence intensity (ordinate) generated by using the protocol described in Example 2 to compare ethidium bromide and PTA 24. The concentration of fluorophore in both cases was 3.08 micromolar. Both curves are normalized to background. Excitation was at 488 nm (534 nm is maximum excitation) and emission was at 625 nm and intensities were measured using a Hitachi F-3010 fluorimeter.

FIGURE 10A is a FACScan™ display (SSC vs FSC) of a typical distribution of white cells lysed with CD4000 WBC DIL without NRBC dye or CEN. The quadrant thresholds were set to preclude the lymphocytes gated on the SSC versus FSC dot plot.

FIGURE 10B is a FACScan™ display of the same blood sample as in FIGURE 10A with unstained CEN added. As can be seen, the unstained CEN demonstrate some FRL3 auto-fluorescence. The region 1 was set to include all FL3+ events in this unstained sample, so that stained cells in the test samples can be counted in the region 2. The region 3 is set to include the CEN population only to measure the mean FL3 of the population.

FIGURE 10C is a FACScan™ display of the same sample lysed with WBC DIL containing 1.0 ug/ml of NRBC dye. 1.3% of FL2+ events were detected in μ L from the gated lymphocytes and 1.08% of FL3+ events are detected from the ungated total white cell population.

FIGURE 10D is a FACScan™ display of the same sample as presented in FIGURE 10C, but with CEN. The region 2 of the FL3 histogram of the ungated population shows the stained CEN population which has the mean FL3 of 3319.8.

5 FIGURE 10E is a FACScan™ display of the same sample lysed in the same diluent as FIGURE 10D, but with 0.5 ug/ml of NRBC dye.

FIGURE 10F is a FACScan™ display of the same sample lysed in the same diluent as FIGURE 10D, but with 0.25 ug/ml of NRBC dye. As can be seen, the stained CEN is still well separated from the white cells.

10 FIGURE 11 is a graphical representation of the efficiency of ³²P radiolabelled plasmid DNA capture onto phenathridinium activated polystyrene microparticles synthesized as described in Example 6. A: initial radioactive counts in solution accounting for the total DNA concentration; B: radioactive counts remaining in solution after removal of DNA via centrifugation as described in Example 6; C: initial radioactive counts on the DNA bound to the microparticle by the phenthridine moiety before release is initiated by NaOH; and D: radioactive counts remaining on the solid after removal of DNA with NaOH.

15 FIGURE 12 is a graphical representation of the efficiency of ³²P radiolabelled plasmid DNA capture onto phenathridinium activated carboxymethyl sepharose beads synthesized as described in Example 6. A: initial radioactive counts in solution accounting for the total DNA concentration; B: radioactive counts remaining in solution after removal of DNA via centrifugation as described in Example 6; C: initial radioactive counts on the DNA bound to the microparticle by the phenthridine moiety before release is initiated by NaOH; and D: radioactive counts remaining on the solid after removal of DNA with NaOH.

20 The present intercalator compounds are substantially monointercalators, versus the polyfunctional (bis) intercalators. The monointercalators according to the present invention are most suitable for application using a multitude of

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intercalators and substituted intercalators which when combined with and functionalized by the various "chains or tails" ("T"), where T is the chain comprised of R, R₁, R₂, R₃, R₄, W, X, Y and Z and bounded by the brackets in the previously discussed formulas, which provide high binding to DNA and RNA and lack of self-quenching and superior transport kinetics. Representative "I" moieties are given in FIGURE 13.

The invention is further defined by the following Examples, which provide basis for the FIGURES and are intended to be illustrative, but not limiting.

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EXAMPLE 1

Synthesis of Phenanthridinium Triamine (PTA) 24 And Precursor Intermediates 20-23

PTA 24, a compound according to the invention, was synthesized through the sequence shown in the schematic as shown in FIGURE 14. The experimental procedures used to obtain product 24 are as illustrated therein.

Intermediate 21. Starting Intermediate 20, 3,8 Diamino 6-phenyl phenathridine (25.0 g, 0.0876 moles), was obtained from the Aldrich Chemical Company (Milwaukee, WI) and added to a single neck 3.0 liter round bottom flask under Argon and equipped with a magnetic stir bar and a reflux condenser. To this vessel, 1.0 liter of dry pyridine was added while stirring. Stirring of the resulting suspension was continued for 15 minutes until all the solid had dissolved. A catalytic amount of N,N-dimethylaminopyridine (1.07 g, 0.0086 moles) was added to this solution while stirring. Acetic anhydride (462 g, 4.9 moles) was then added and the resulting reaction mixture was refluxed for 8-12 hours. The reaction mixture was then allowed to cool and the solvent was removed in vacuo. For purification of the Product II, a gradient silica gel column was performed using 2.0 liters of 45/40/10/5 Ethyl acetate/hexane/ CH₂Cl₂/CH₃OH followed by 1.0 liter of 40/40/10/10

EtOAc/hexane/ CH₂Cl₂/CH₃OH. Fractions of 10.0 ml were collected and appropriate fractions were recombined and the solvent was removed in vacuo. The sticky gum-like residue was then dissolved in hot EtOH (220 ml) and precipitated by cooling to 0° C. The mother liquor was decanted off and 200
5 ml of fresh EtOH was added. The solid was redissolved by heating and allowed to crystallize at -4° C for 48 hours. Crystals were collected from both the mother liquor and the second recrystallization and were washed with a small amount of cold EtOH and dried under high vacuum for several hours. Isolated yield of pure product after column chromatography and two
10 recrystallizations was 32%. ¹H NMR CD₃OD (300 MHz) 9.1 (d, 1H 8.82 Hz), 9.0 (d, 1H, 8.75 Hz), 8.08 (s, 1H), 7.95 (s, 1H), 7.92 (d, 1H, 4.5 Hz), 7.8 (m, 3H), 7.65 (m, 3H), 2.45 (s, 6H), 2.35 (s, 6H); ¹³C NMR CD₃OD (75.45 MHz) 174.5, 163.7, 145.3, 142.1, 140.6, 139.9, 134.4, 133.8, 130.8, 130.6, 129.8, 127.3, 125.9, 125.6, 124.8, 27.1, Exact Mass Calc. for C₂₇H₂₃N₃O₄,
15 Calc. 453.1688 exact mass, Obs: 453.1683; CH analysis Calc for C₂₇H₂₃N₃O₄ C: 71.51 H: 5.11 N: 9.27 Found C: 71.77 H: 5.10 N:9.20.

Intermediate 22. Intermediate 22 was synthesized from the diamide 21 via a modification of a literature procedure (Gaugain et al., Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078) for quarternization of the diamide of 3,8
20 Diamino-6-phenyl phenanthridine. Diamide 21 (10.5 g, 0.023 moles) was placed in a 2.0 liter round bottom flask under argon and equipped with a magnetic stir bar and reflux condenser. 1,3 Dibromopropane (1.0 liter, 9.86 moles) was added to this flask and the resultant mixture was brought to reflux for 7 hours. The solution was cooled overnight and the precipitant was filtered
25 and washed with diethyl ether. Obtained 10.44 g (68.7%) of crude material 22. This material was recrystallized from CH₃OH to yield 5.3 g of diacetyl bromide 22. ¹H NMR CD₃OD (300 MHz) delta 10.75 (s, 1H), 10.45 (s, 1H), 9.3 (s, 1H), 9.09 (d, 9.2 Hz, 1H), 9.04 (d, 9.2 Hz, 1H), 8.45 (d, 9.1 Hz, 2.2 Hz, 1H), 8.12 (s, 1H), 8.07 (d 9.0 Hz, 1H), 7.95 (m, 3H), 7.85 (m, 3H), 5.0
30 (t, 9.0 Hz, 3H), 3.6 (t, 6.0 Hz, 2H), 2.65, 2.38 7.75 ¹³C NMR ₂₆DMSO [????]

(75.45 MHz) delta 169.9, 169.3, 163.8, 142.1, 139.9, 134.2, 131.5, 130.5, 129.5, 128.4, 125.9, 125.4, 123.6, 122.3, 121.6, 119.0, 107.7, 55.7, 30.7, 24.5, 24.2; exact mass Calc. for $C_{26}H_{29}N_3O_2Br_2$ free salt (FAB+) 490.1131 Obs: 490.1139; CH analysis Calc. for $C_{26}H_{29}N_3O_2Br_2$ H: 4.41 C: 54.66 N:7.36
5 Found H: 4.25 C: 54.65 N: 7.30.

Intermediate 23. Intermediate 22 (5.3 g, 0.0093 moles) was added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) was then added to this flask while stirring under nitrogen and diethylene triamine (29.2 g, 0.283 moles) was added while stirring was
10 continued. The resultant transparent solution was heated to reflux overnight under nitrogen. This solution was then allowed to cool to room temperature and poured into distilled H_2O . Then this mixture was concentrated in vacuo until only the H_2O remained. An additional 50-75 ml H_2O was added and the solution was allowed to cool to $0^\circ C$. The solid was then filtered and washed
15 with ice cold water. This material was then redissolved in EtOH and precipitated with 10 N HCl. After filtration of the suspension, the resultant solid was recrystallized from hot ethanol upon cooling to $0^\circ C$ for 15 minutes. A second crop was also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids were then
20 combined and the final product 23 (2.5 g) was obtained after high vacuum overnight. 1H NMR CD_3OD (300 MHz) delta 9.2 (s, 1H), 9.05 (d, 1H), 8.95 (d, 1H), 8.4 (broad s, 2H), 8.3 (broad s, 1H), 7.9 (broad m, 3H), 7.7 (broad m, 2H), 5.1 (broad m, 1H), 3.9 (broad s, 3H), 3.45 (broad m, 2H), 3.85 (broad m, 2H), 2.5 (broad m, 3H), 2.3 (broad m, 3H); MS Calc. for free salt
25 $C_{30}H_{37}N_6O_2$ (FAB+) 513 Obs: 513.

Product 24. Synthesis and purification of the phenanthridinium triamine, PTA, 24 was accomplished by the following protocol. Triamine 23 (2.35 g, 0.0036 moles) was dissolved in 75.0 ml methanol and 75 ml of 4N HCl was added. The mixture was refluxed for 2 hours and allowed to cool. Ethanol
30 was added to this solution and resulting precipitate was filtered and washed

with a minimal amount of cold ethanol. The filtrate was reconcentrated and fresh ethanol and concentrated aqueous HCl was added. This resulting precipitate was also filtered. Next, this filtrate was concentrated to near dryness and Et₂O was added and the solid filtered off. The last remaining
5 unfilterable residue was then dissolved in concentrated HCl and precipitated with EtOH. This material was filtered and washed with ethanol, combined all solid materials from the above sequence and subjected this material to high vacuum overnight to obtain 2.03 g total of the high affinity fluorescent DNA stain PTA 24. ¹H NMR d₆-DMSO (300 MHz) delta 10.0 (broad s, 2H), 9.65
10 (broad s, 2H), 8.68 (d, 14.2 Hz, 2H), 8.35 (broad s, 2H), 7.75 (m, 4H), 7.65 (s, 1H) 7.55 (d, 9.2 Hz, 2H), 7.35 (d, 9.2 Hz, 2H), 6.28 (s, 1H), 4.5 (broad s, 2H), 4.0 (broad s, 8H), 3.4 (broad s, 2H), 3.0 (broad s, 2H), 2.3 (broad m, 2H); ¹³C NMR d₆-DMSO (75.45 MHz) delta 159.7, 151.1, 134.6, 131.7, 130.9, 129.4, 128.8, 128.4, 124.9, 122.9, 120.1, 117.4, 99.6, 51.4, 43.8, 42.5,
15 40.3, 34.9, 18.5; High resolution mass spec. C₂₆H₃₃N₆(FAB+) Calc. 429.2767, Obs: 429.2766; CH analysis Calc for C₂₆H₃₇N₆Cl₄.3H₂O was H 6.89 C 49.61 N 13.35 Found H: 6.16; C 49.74 N: 13.08.

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EXAMPLE 2

Hybridization Assay

PTA 24 was used to quantitate hybridization when a target oligonucleotide was titrated with its complementary partner. A comparison of ethidium bromide staining versus PTA 24 for detecting this hybridization, as
25 per the following protocol, can be found in FIGURE 9 and the comparison of ethidium homodimer versus PTA 24, as per the following protocol, can be found in FIGURE 8. Complementary strands of DNA (oligodeoxythymidylic acid, d(pT), and oligodeoxyadenylic acid (d(pA),) were obtained from the Sigma Chemical Co. in St. Louis, MO. A stock solution of d(pA)₉ was made
30 at 5 units/ml of 0.05M TRIS, 0.2N NaCl, 1MM EDTA, pH 8.4. For polyA,

$\epsilon=8.4$ AU/mM cm or $8,400$ M⁻¹cm⁻¹; therefore, with 9 base pairs for d(pA)9, the ϵ is $75,600$ M⁻¹cm⁻¹. This stock was then diluted 10x to obtain stock at 6.61×10^{-6} M, or 6.6 μ M. The d(pT)₉ stock was made at 25 units/5.0 ml and used for titration without further dilution in the same buffer. Since the ϵ for polyT is 8.15 AU/mM cm or $8,150$ M⁻¹cm⁻¹ per base pair, or $73,350$ M⁻¹cm⁻¹ per oligo, the concentration of the oligo stock was 68 μ M in DNA molecules. A titration was performed using a Hitachi F-4010 Fluorescence Spectrophotometer equipped with 0.5 ml microcells to obtain fully corrected spectra and an excitation wavelength of 488-550 nm (optimal around 534) and an emission wavelength of 600-650 nm (optimal around 625). Equivalents of d(pT)₉ were added at the following increments: 0.02, 0.05, 0.080, 0.150, 0.300, 0.500, 0.700, 1.00, 2.00, 5.00 equivalents. Each sample in the titration curve was prepared individually by dividing the initial d(pA)9 stock into 10 x 1.0 ml aliquots. The addition of complement was then accomplished by micropipetting an appropriate amount (2, 5, 8, 15, 30, 50, 70, 100, 200, and 500 μ l, respectively) of d(pT)₉ stock to each of a series of the 10 aliquots. Each aliquot, obtaining progressively larger molar ratios of the two complementary strands, was incubated at ambient temperature for 15 minutes, the dye was added as 20.0 μ l aliquots of a 154 μ M solution of the dye in 0.05M TRIS, 0.2N NaCl, 1mM EDTA, pH 8.4 buffer. This corresponds to a dye/DNA b.p. ratio of 1/20 at saturation with complementary oligo. Overall concentrations of dye and oligo vary in the saturation plot because of the use of varied increments additions from the same stock solution. After an additional 15 minute incubation time, the relative fluorescence intensity was then read at 625 nm and recorded to generate a standard curve which is directly proportional to the quantity of dsDNA hybridization, or target sequence, under these conditions. The background fluorescence, or initial residual fluorescence, is then subtracted out as a constant for all curves for comparison of the various titration curves on the same graph.

EXAMPLE 3

Gel Electrophoresis Application

An agarose gel was run to compare the staining intensity of ethidium bromide (Aldrich Chemical Co., Milwaukee, WI), ethidium homodimer -1 (Molecular Probes, Cat. # E 1169, Eugene, Oregon), and PTA 24 stain. Plasmid, pBR322, at 2.1 mg in 7 ml stock was incubated at 37°C for 1 hour with 1 ml of BAMH restriction enzyme with 2 ml 10X React2 Buffer and diluted to 20 ml total with 10 ml H₂O. This mixture was then used to prepare 3 stocks of nicked pBR322 plasmid at 0.63 mg per 6 ml for each vial. Each of these stocks were diluted further with H₂O and 20% glycerol to final DNA stocks of 20 ng/ml, 800 pg/ml, 160 pg/ml, and 40 pg/ml with a 1:4 ratio of dye to DNA base pairs in each for a total of 12 stocks. A 5 ml aliquot of each stock was loaded into 12 separate lanes in agarose gel and electrophoresis was run for 30 minutes in 4 mM TRIS, pH8.2, with 0.01mM EDTA buffer. The gel was then removed and photographed under exposure to U.V. light in a conventional gel box.

EXAMPLE 4

Protocol For Synthesis Of Intercalator Activated Carboxymethyl Styrene Microparticle Capture Reagent

The synthesis of intercalator derivatized solid phase microparticle (MP) capture reagent was accomplished by the scheme depicted in the following schematic and effected by the following procedure:

A 45 aliquot of $0.275 \pm \mu\text{m}$ microparticles (Seradyne, Indianapolis, IN) were placed in a 4 ml vial and the surfactant was exchanged out using Bio-Rex 501-D ion exchange mixed bed resin (Bio-Rad, Richmond, CA). After gentle shaking for 2 hours, the resin was filtered out from the mixture by using a coarse fritted glass funnel equipped with a reduced pressure collection chamber. The sample was diluted to a concentration of MP at 10% solids by weight.

The total amount of equivalents of reactive carboxylic acid were calculated from the titration specifications of the vendor.

5 A stock solution of sulfo N-hydroxysuccinimide (Pierce, Rockford, IL) was made at 11 mg/ml (20 mM) in H₂O and a stock solution of EDAC (Sigma Chemical Co., St. Louis, MO) at 10 mg/ml (5 mM) was made in H₂O. Five equivalents of EDAC (290 μ l stock) was added to the carboxymicroparticle reaction mixture, followed by 5.0 equivalents of sulfo N-hydroxysuccinimide (330 μ l stock). This mixture was allowed to incubate at room temperature for 2 hours and then a 2.0 molar equivalent of PTA 24 (4 mg) was added at a
10 concentration of 8 mg/400 μ l, or 2.0 mg/100 μ l in pH 8.0 0.1 N NaCl 0.1N Pi phosphate buffer. N-hydroxysuccinimide (Pierce) can be substituted for sulfo N-hydroxysuccinimide if it is first dissolved in a stock of DMF (Dimethyl formamide) and aliquoted as described above. After allowing 24 hours for complete reaction, the free dye was then removed by centrifugation,
15 removal of mother liquor, and resuspension for several attempts until the solution went clear and no more dye was extracted from the samples. The purified capture reagent was then diluted to a stock of 2-4 % solids in H₂O.

A general schematic representation of this Example is given in FIGURE
15.

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EXAMPLE 5

Solid Phase DNA Capture

CM (carboxy modified) Sepharose was obtained from Sigma Chemical
25 Co. (St. Louis, MO) in an ethanol/H₂O mixture. The solution was estimated at 50% solids based on total volume occupied by the solid and liquid portions on extended standing. This suspension was then mixed uniformly and diluted to 10% solids. 200 μ l of this stock was removed and calculated at 0.12 meq/gram to be 0.012 meq of acid total. Stock of EDAC and N-
30 hydroxysuccinimide were prepared and 5.0 equivalents of each activating

reagent were added to this suspension. For this preparation, 13.2 mg (in 1.32 ml) HOSuc and 11.25 mg EDAC (in 1.02 ml) were used and 8.0 mg total of the PTA 24 intercalator. After incubation for 2-24 hours, the suspensions were then cleaned by repeated washing and gentle centrifugation steps until no more color was removed from the solid upon dilution. A stock was prepared at 10% solids in H₂O. Note that controls were run with PTA 24 modified and non-modified solid phases and minimal non-specific capture of DNA occurred with the underivatized materials.

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EXAMPLE 6

Protocol For DNA Capture By Intercalator Modified Solid Phase

1. Place 50 μ l of activated microparticles in a 1.5 ml eppendorf.
2. Add 150 μ l of PBS and 1-20 u of a 5.0 kb linearized plasmid end-labeled with ³²P. Alternatively, added 1-50 μ l of biological sample or another purified DNA.
3. Mix by rotation for one hour at ambient temperature.
4. Pellet the microparticles by centrifugation at 5,000 rpm for 5 minutes.
5. Wash one or two times with 200 μ l of PBS.
6. Release the DNA by adding 50 μ l of 0.5 M NaOH and mix for 15 minutes at ambient temperature.
7. Centrifuged and collected the supernatant, which contains released DNA.

The efficiency of DNA capture was measured using ³²P radiolabelled plasmid DNA in the above-described protocol. The results are found in FIGURE 11 using the intercalator modified polystyrene microparticles prepared as per Example 4 and FIGURE 12 and using the intercalator modified CM Sepharose beads prepared per Example 5. The data indicates that the DNA

binding to the intercalator modified solid phase was specific and induced by the covalent attachment of intercalator 24 to the solid phase.

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EXAMPLE 7

Relative Staining Intensities Of Ethidium Homodimer And Phenanthridinium Triamine 24 In A Flow Cytometric Study Of Chicken Erythrocyte Nuclei (CEN)

Protocol: 50 μ l of whole blood sample from two in-house donors and
10 3 μ l of CEN suspension was added to 1.0 ml of pre-warmed at 40°C WBC DIL
without and with the NRBC dye at 1 μ g/ml concentration, mixed, introduced
to the FACScan™ and 20" readings were acquired. Chicken erythrocyte nuclei
(CEN) were used to measure the brightness of the FL3 staining (mean FL3 of
CEN). The whole blood samples used were about 4-5 hours old. The data for
15 this experiment is shown in FIGURES 1-6.

EXAMPLE 8

Comparative Performance Of Reduced Phenanthridinium Triamine 20 24 Dye Concentrations Relative To Ethidium Homodimer

The effect of a reduction in PTA 24 dye concentration (FIGURES 10A-F) relative to ethidium homodimer was demonstrated as follows:

Method: The experiment was designed to show the correlation between
the dye concentration and the percent of FL2+ events in the UL quadrant on
25 the FL1 versus FL2 dot plots. The WBC DIL used contained 0.5%
weight/volume of ammonium chloride, 0.075% of volume of formaldehyde,
0.01% weight/volume of saponin, 0.01% weight/volume of potassium
bicarbonate, and 20 mM acetate buffer with a pH of about 6.0 and an
osmolality of about 270 mOsm per liter. 50 μ l of whole blood sample from
30 each of two in-house donors was added to 1.0 ml of pre-warmed at 40°C WBC
DIL without and with the NRBC dye of varying concentration (0.25, 0.50,

0.75, and 1.0 ug/ml), mixed, introduced to the FACScan™ and 20" readings were acquired. Chicken erythrocyte nuclei (CEN) suspension was used to measure the brightness of the FL3 staining (mean FL3 of CEN). The whole blood samples used for this experiment were about 6 hours old.

5 It was observed that the CEN DNA is essentially indistinguishable from the background without the PTA 24 (FIGURE 10B) and that the dye concentration can be reduced to 75 % of that of ethidium homodimer (FIGURE 5) and still maintain acceptable separation from the background (FIGURE 10F). Such a reduction can lead to significantly reduced non-specific binding
10 and substantial savings in dye usage.

EXAMPLE 9

Viability Dyes On The Coulter Elite™ Flow Cytometer

15 Cell Isolation Protocol: Each tube of ficol isolated cells were treated as follows: PBS with 0.1 % NaAzide and 1.0% albumin (Sigma catalogue #1000-3) Ficol specific gravity 1.119 (Sigma Histopaque catalogue #1119-1).

10 ml of whole blood (EDTA anticoagulant) was diluted with 10 ml of PBSW. Into 4, 15 ml conical bottom tubes, 5 ml of the diluted blood was
20 layered over 5 ml of ficol. The tubes were spun for 30 minutes at 400 x G. The interface layer which contains the lymphocytes, monocytes, granulocytes and platelets was aspirated and washed once in 5 ml PBS, by centrifuging tubes at 300 x G for 6 minutes. The cell pellet was resuspended in PBS, cells counted, and adjusted to 8.5×10^6 cells per ml.

25

Cell Staining Protocol:

Dye solutions:

PTA 24 - Stock solution 10 ug/ml made by dissolving PTA 24 in PBS with 0.1 % NaAzide.

Propidium iodide (P.I.) - Stock solution 0.5 mg/ml made by dissolving P.I. in PBS with 0.1 % NaAzide.

P.I. Staining:

5 In 12 x 75 mm tube, 117.6 μ l of cells was mixed gently with 14.7 μ l of P.I. stock dye solution. After 20 seconds, the tube was placed on a Coulter Elite™ flow cytometer and data collected.

Procedure from "Discrimination of Viable and Non-Viable Cells Using Prodidium Iodide in Two Color Immunofluorescence", Cytometry by Sasaki
10 et al., Vol. 8, 1987, pp. 413-420.

PTA 24 Staining:

In 12 x 75 mm tube, 23.5 μ l of cells was gently mixed with 76 μ l of PTA 24
15 stock dye solution. After 20 seconds, the tube was placed on a Coulter Elite™ flow cytometer and data collected.

Trypan Blue Staining:

In 12 x 75 mm tube, 5 μ l of working solution Trypan Blue and 5 μ l of cells
20 were gently mixed and cells counted in a mehaycytometer using standard white light illumination. A minimum of 500 cells were counted within 3 minutes of staining.

Procedure from Selected Methods in Immunology by Mishell and Shiigi, 1980,
pp. 16-17.

25

Flow cytometer protocol: Cells analyzed on the Elite™ flow cytometer (Coulter Electronics, Inc.).

Samples were excited with an argon laser at 488 nm and 15 mW of power. Data was gated on the basis of size and granularity to exclude red
30 blood cells, platelets and debris. The linear dye fluorescence of the gated distribution was analyzed using unstained cells as a control. The percent

positive events (dead cells) and the mean fluorescence of the dead cell distribution were recorded.

TABLE 1

5	Viability Dyes on the Coulter Elite™ Cytometer		
	Time Point	Sample	% Positive (Dead Cells)
	5 hr	P.I.	2.5
		PTA	2.2
		Trypan Blue	1.4
10	27 hr	P.I.	6.3
		PTA	7.7
		Trypan Blue	4.8
	103 hr	P.I.	26.8
		PTA	19.1
15		Trypan Blue	10.2

EXAMPLE 10

Synthesis Of Compound 25

20 Compound 22 (0.075g, 0.00013 moles) was added to a 50 ml round bottom flask equipped with a magnetic stir bar and reflux condensor. Methanol (10 ml) was then added to this flask while stirring under nitrogen and tris(2-aminoethyl)amine (1.016 g, 0.00518 moles) was added while stirring was continued. The resultant transparent solution was heated to

25 reflux overnight under nitrogen. This solution was then allowed to cool to room temperature and poured into distilled H₂O. Then this mixture was concentrated in vacuo until only the H₂O remained. An additional 50-75 ml H₂O was added and the solution was allowed to cool to 0°C. The solid was then filtered and washed with ice cold water. This material was then

30 redissolved in EtOH and precipitated with 10 N HCl. After filtration of the

suspension, the resultant solid was recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop was also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids were then combined and the final product 23 (2.5 g) was obtained after high vacuum overnight. Material was then carried through to the next step of hydrolysis.

The amine residue from above was dissolved in 10.0 ml methanol and 15 ml of 4N HCl was added. The mixture was refluxed for 2 hours and allowed to cool. Ethanol was added to this solution and resulting precipitate was filtered and washed with a minimal amount of cold ethanol. The filtrate was reconcentrated and fresh ethanol and concentrated aqueous HCl was added. This resulting precipitate was also filtered. Next, this filtrate was concentrated to near dryness and Et₂O was added and the solid filtered off. The last remaining unfilterable residue was then dissolved in concentrated HCl and precipitated with EtOH. This material was filtered and washed with ethanol, combined all solid materials from the above sequence and subjected this material to high vacuum overnight to obtain the of the high affinity fluorescent DNA stain phenathridium amine derivative 25. High resolution mass spec. C₂₈H₃₈N₇(FAB+) Calc. 472.3189 Obs: 472.3191.

The structural formula of compound 25 is shown in FIGURE 16.

EXAMPLE 11

Synthesis Of Compound 26

Intermediate 22 (0.2 g, 0.406 mmoles) was added to a 50 ml round bottom flask equipped with a magnetic stir bar and reflux condensor. Methanol (10 ml) was then added to this flask while stirring under nitrogen and 1,4 Bisamino(3-aminopropyl)piperazine (3.3 g, 16.26 mmoles) was added while stirring was continued. The resultant transparent solution was heated to reflux overnight under nitrogen. This solution was then allowed to

cool to room temperature and poured into distilled H₂O. Then this mixture was concentrated in vacuo until only the H₂O remained. An additional 50-75 ml H₂O was added and the solution was allowed to cool to 0°C. The solid was then filtered and washed with ice cold water. This material was then redissolved in EtOH and precipitated with 10 N HCl. After filtration of the suspension, the resultant solid was recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop was also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids were then combined and the diamine amine product was obtained after high vacuum overnight. Material was then carried through to the next step of hydrolysis.

The amine residue from above was dissolved in 10.0 ml methanol and 15 ml of 4N HCl was added. The mixture was refluxed for 2 hours and allowed to cool. Ethanol was added to this solution and resulting precipitate was filtered and washed with a minimal amount of cold ethanol. The filtrate was reconcentrated and fresh ethanol and concentrated aqueous HCl was added. This resulting precipitate was also filtered. Next, this filtrate was concentrated to near dryness and Et₂O was added and the solid filtered off. The last remaining unfilterable residue was then dissolved in concentrated HCl and precipitated with EtOH. This material was filtered and washed with ethanol, combined all solid materials from the above sequence and subjected this material to high vacuum overnight to obtain the of the high affinity fluorescent DNA stain phenathridium amine derivative 26. Mass Spec. C₂₆H₃₀N₅(FAB+) Calc. 412, Obs: 412.

The structural formula of compound 26 is shown in FIGURE 16.

EXAMPLE 12Synthesis of Compound 27

Intermediate 22 (0.06 g, 0.12 mmoles) was added to a 50 ml round bottom flask equipped with a magnetic stir bar and reflux condensor.

5 Methanol (10 ml) was then added to this flask while stirring under nitrogen and piperazine-1-carboxyaldehyde (1.37 g, 12.0 mmoles) was added while stirring was continued. The resultant transparent solution was heated to reflux overnight under nitrogen. This solution was then allowed to cool to room temperature and poured into distilled H₂O. Then this mixture was
10 concentrated in vacuo until only the H₂O remained. An additional 50-75 ml H₂O was added and the solution was allowed to cool to 0°C. The solid was then filtered and washed with ice cold water. This material was then redissolved in EtOH and precipitated with 10 N HCl. After filtration of the suspension, the resultant solid was recrystallized from hot ethanol upon
15 cooling to 0°C for 15 minutes. A second crop was also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids were then combined and the diamine amine product was obtained after high vacuum overnight. Material was then carried through to the next step of hydrolysis.

20 The amine residue from above was dissolved in 10.0 ml methanol and 15 ml of 4N HCl was added. The mixture was refluxed for 2 hours and allowed to cool. Ethanol was added to this solution and resulting precipitate was filtered and washed with a minimal amount of cold ethanol. The filtrate was reconcentrated and fresh ethanol and concentrated aqueous HCl was
25 added. This resulting precipitate was also filtered. Next, this filtrate was concentrated to near dryness and Et₂O was added and the solid filtered off. The last remaining unfilterable residue was then dissolved in concentrated HCl and precipitated with EtOH. This material was filtered and washed with ethanol, combined all solid materials from the above sequence and
30 subjected this material to high vacuum overnight to obtain the of the high

affinity fluorescent DNA stain phenathridium amine derivative 27. Mass Spec. $C_{32}H_{44}N_7$ (FAB+) Calc. 526, Obs: 526.

The structural formula of compound 27 is shown in FIGURE 16.

5

EXAMPLE 13

Synthesis Of Compounds 28a-42a And Their Related Compounds

The reaction scheme for the general synthesis of compounds 28a-42a, and their respective precursors, is given in FIGURE 17.

10 Generally, compound 22 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and an appropriate amine (0.283 moles) is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This
15 solution is then allowed to cool to room temperature and is poured onto distilled H_2O . Then, this mixture is concentrated in vacuo until only the H_2O remains. An additional 50-75 ml H_2O is added and the reaction mixture is cooled to $0^\circ C$. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N
20 HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to $0^\circ C$ for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product, depending on the amine used to carry out the reaction, is
25 obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to
30 those skilled in the art.

Listed below are the products that can be obtained from their corresponding starting amines.

- 4-amino-1-benzylpiperidine to yield compound 28a
spermine to yield compound 29a
5 pyridine to yield compound 30a
2-(2-Aminoethyl)-1-methylpyrrolidine to yield compound 31a
1-(2-Aminoethyl)pyrrolidine to yield compound 32a
1-(2-Aminoethyl)piperidine to yield compound 33a
2-(2-Aminoethyl)pyridine to yield compound 34a
10 1-(2-Aminoethyl)piperazine to yield compound 35a
4-(2-Aminoethyl)morpholine to yield compound 36a
1-Amino4-(2-hydroxyethyl)piperazine to yield 37a
4-(Aminomethyl)piperidine to yield 38a
2-(Aminomethyl)pyridine to yield 39a
15 aniline to yield 40a
1-(3-Aminopropyl)imidazole to yield 41a
4-(3-Aminopropyl)morpholine to yield 42a

- Synthesis and purification of the final products 28b-42b, depending
20 on the starting amide used, 28a-42a, can be accomplished by the following
general procedure. The appropriate amide (0.0036 moles) is dissolved in
75.0 ml methanol and 75 ml of 4N HCl is added. The mixture is refluxed
for 2 hours and allowed to cool. Ethanol is added to this solution and
resulting precipitate is filtered and washed with a minimal amount of cold
25 ethanol. The filtrate is reconcentrated and fresh ethanol and concentrated
aqueous HCl is added. This resulting precipitate is also filtered. Next, this
filtrate is concentrated to near dryness and Et₂O is added and the solid
filtered off. The last remaining unfilterable residue is then dissolved in
concentrated HCl and precipitated with EtOH. This material is filtered and
30 washed with Ethanol. All solid materials are combined from the above

sequence and is subjected to high vacuum overnight to obtain the high affinity fluorescent DNA stain 1b-15b, depending on the amine used as described above. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Listed below are the products that can be obtained from their corresponding starting amides.

- | | |
|----|--|
| 10 | amide <u>28a</u> to yield product <u>28b</u> |
| | amide <u>29a</u> to yield product <u>29b</u> |
| | amide <u>30a</u> to yield product <u>30b</u> |
| | amide <u>31a</u> to yield product <u>31b</u> |
| | amide <u>32a</u> to yield product <u>32b</u> |
| 15 | amide <u>33a</u> to yield product <u>33b</u> |
| | amide <u>34a</u> to yield product <u>34b</u> |
| | amide <u>35a</u> to yield product <u>35b</u> |
| | amide <u>36a</u> to yield product <u>36b</u> |
| | amide <u>37a</u> to yield product <u>37b</u> |
| 20 | amide <u>38a</u> to yield product <u>38b</u> |
| | amide <u>39a</u> to yield product <u>39b</u> |
| | amide <u>40a</u> to yield product <u>40b</u> |
| | amide <u>41a</u> to yield product <u>41b</u> |
| | amide <u>42a</u> to yield product <u>42b</u> |

25

EXAMPLE 14

Synthesis Of Compound 50 And Its Related Compounds

The reaction scheme for the general synthesis of compound 50 and its precursors is given in FIGURE 18.

30

2-Methylbenzathiazole 43, from the Aldrich Chemical Company (Milwaukee, WI), is alkylated to produce compound 44 using methyl iodide by adapting procedures such as found in P.L. Southwick and A.S. Waggoner et al., US Patent No. 4,981,977, Jan. 1, 1991, or in Ernst et al., Cytometry, 10, 1989, pp. 3-10. Compound 45 is obtained from the Aldrich Chemical Company and is reacted with 3-bromo-1-propanol (also available from the Aldrich Chemical Company) adapting procedures of Gaugain, et al, Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078. The condensation of compound 44 and 46 is effected by adapting procedures found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and Related Compounds", Wiley, 1964, pg. 37 to yield compound 47. Compound 48 can then be obtained by converting the alcohol to the tosylate by using procedures such as found in Wiberg, K. et al., J. Am. Chem. Soc., 92 (3), 1970, pp. 553-564. The tosylate 48 is then converted to the bromide via a nucleophilic displacement reaction with sodium bromide as per Wilt, J., J. Org. Chem., 35 (8), 1970, pp.2803-2806 to yield compound 49. Alternatively, a one step procedure is provided in Hooz, J. et al., Can. J. Chem., 46, 1968, pp. 86-87. Compound 49 can then be reacted with diethylene triamine (available from the Aldrich Chemical Company) as per the following procedure.

Compound 49 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the diethylene triamine (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then

redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 50 is obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Amine derivatives of compound 49 can be synthesized as follows. Compound 49 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condensor. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the appropriate amine selected from the following list (0.283 moles), which are available from the Aldrich Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 49a-49o can be obtained and is subjected to high vacuum overnight.

Listed below are the products that can be obtained from their corresponding amines.

- 4-amino-1-benzylpiperidine to yield 49a
spermine to yield to yield 49b
5 pyridine to yield to yield 49c
2-(2-Aminoethyl)-1-methylpyrrolidine to yield 49d
1-(2-Aminoethyl)pyrrolidine to yield 49e
1-(2-Aminoethyl)piperidine to yield 49f
2-(2-Aminoethyl)pyridine to yield 49g
10 1-(2-Aminoethyl)piperazine to yield 49h
4-(2-Aminoethyl)morpholine to yield 49i
1-Amino4-(2-hydroxyethyl)piperazine to yield 49j
4-(Aminomethyl)piperidine to yield 49k
2-(Aminomethyl)pyridine to yield 49l
15 aniline to yield 49m
1-(3-Aminopropyl)imidazole to yield 49n
4-(3-Aminopropyl)morpholine to yield 49o

20 Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

25 Since the structures of compounds 49a-49o are unambiguous from the above generic procedures, there structures are not given.

EXAMPLE 15

Synthesis Of Compounds 55 And 55a-55b

The reaction scheme for the general synthesis of compounds 55, 55a, and their precursors, is given in FIGURE 19. The structural formula of compounds 55a-55p are given in FIGURE 20.

2-Methylbenzathiazole, 43, is obtained from the Aldrich Chemical Company (Milwaukee, WI). It is alkylated to produce compound 51 using 3-bromo-1-propanol adapting procedures such as found in Gaugain et al., Biochemistry, 17 (24), 1978, 5071-5078. Compound 45 is obtained from the Aldrich Chemical Company and is reacted with 3-bromo-1-propanol (also available from the Aldrich Chemical Company) by also adapting procedures such as found in Gaugain et al., Biochemistry 17 (24) 1978, 5071-5078. The condensation of compound 51 and 52 is effected by adapting procedures found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and Related Compounds", Wiley, 1964, pg. 37 to yield compound 52. Compound 53 is then obtained by converting the alcohol to the tosylate by using procedures such as found in Wiberg, K. et al., J. Am. Chem. Soc., 92 (3), 1970, pp. 553-564. The tosylate 53 is then converted to the bromide via a nucleophilic displacement reaction with sodium bromide as per Wilt, J., J. Org. Chem., 35 (8), 1970, pp. 2803-2806 to yield compound 54.

Alternatively, a one step procedure is provided in Hooz, J. et al., Can. J. Chem., 46, 1968, pp. 86-87.

Compound 54 can then be reacted with diethylene triamine or other appropriate amine from the following list (available from the Aldrich Chemical company) to yield compounds 54a-54p in accordance with the following procedure.

Compound 54 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to the flask while stirring under nitrogen and the

diethylene triamine or other appropriate base (0.283 moles), available from the Aldrich Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 9a-p is obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

As long as the ratios of reagents and solvents are held constant, one can scale up or down the amounts of reagents using the method of ratio and proportions well known to those skilled in the art.

Listed below are the products that can be obtained from their corresponding starting amines.

- diethylene triamine to yield compound 54a
- 4-amino-1-benzylpiperidine to yield compound 54b
- spermine to yield compound 54c
- pyridine to yield compound 54d
- 2-(2-Aminoethyl)-1-methylpyrrolidine to yield compound 54e
- 1-(2-Aminoethyl)pyrrolidine to yield compound 54f
- 1-(2-Aminoethyl)piperidine to yield compound 54g

2-(2-Aminoethyl)pyridine to yield compound 54h
1-(2-Aminoethyl)piperazine to yield compound 54i
4-(2-Aminoethyl)morpholine to yield compound 54j
1-Amino4-(2-hydroxyethyl)piperazine to yield 54k
5 4-(Aminomethyl)piperidine to yield 54l
2-(Aminomethyl)pyridine to yield 54m
aniline to yield 54n
1-(3-Aminopropyl)imidazole to yield 54o
4-(3-Aminopropyl)morpholine to yield 54p

10

EXAMPLE 16

Synthesis Of Compounds 58 And 58a-58p

The reaction scheme for the general synthesis of compounds 58, 58a
15 and their precursors, is given in FIGURE 21. The structural formula of
compounds 58a-58p are given in FIGURE 22.

2-Methylbenzathiazole 43 is obtained from the Aldrich Chemical
Company (Milwaukee, WI). It is alkylated to produce compound 51 using
3-bromo-1-propanol by adapting procedures Gaugain, et. al, Biochemistry,
20 Vol. 17, No. 24, 1978, pp. 5071-5078. Compound 45 is obtained from the
Aldrich Chemical Company and is reacted with methyl iodide (also available
from the Aldrich Chemical Company) to yield compound 55 using
procedures such as found in Southwick et al. US Patent No. 4,981,977, Jan.
1, 1991, or in Ernst et al., Cytometry, 10, 1989, pp. 3-10. The
25 condensation of compound 51 and 55 is effected by adapting procedures
found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and
Related Compounds", Wiley, 1964, pg. 37 to yield compound 56.
Compound 57 is then obtained by converting the alcohol to the tosylate by
using procedures such as found in Wiberg, K. et al., J. Am. Chem. Soc.,
30 92 (3), 1970, pp. 553-564. The tosylate 57 is then converted to the

bromide via a nucleophilic displacement reaction with sodium bromide described by Wilt, J., J. Org. Chem., 35 (8), 1970, pp. 2803-2806 to yield compound 58.

Alternatively, a one step procedure can be used as provided in Hooz, J. et al., Can. J. Chem., 46, 1968, pp. 86-87.

Compound 58 can then be reacted with diethylene triamine or other appropriate amine (available from the Aldrich Chemical company) as follows.

Compound 58 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the diethylene triamine or other appropriate amine (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 58a-58p is obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

As long as the ratios of reagents and solvents are held constant, one can scale up or down the amounts of reagents using the method of ratio and proportions well known to those skilled in the art.

Listed below are the products that can be obtained from their
5 corresponding amines.

- diethylene triamine to yield compound 58a
- 4-amino-1-benzylpiperidine to yield compound 58b
- spermine to yield compound 58c
- pyridine to yield compound 58d
- 10 2-(2-Aminoethyl)-1-methylpyrrolidine to yield compound 58e
- 1-(2-Aminoethyl)pyrrolidine to yield compound 58f
- 1-(2-Aminoethyl)piperidine to yield compound 58g
- 2-(2-Aminoethyl)pyridine to yield compound 58h
- 1-(2-Aminoethyl)piperazine to yield compound 58i
- 15 4-(2-Aminoethyl)morpholine to yield compound 58j
- 1-Amino4-(2-hydroxyethyl)piperazine to yield 58k
- 4-(Aminomethyl)piperidine to yield 58l
- 2-(Aminomethyl)pyridine to yield 58m
- aniline to yield 58n
- 20 1-(3-Aminopropyl)imidazole to yield 58o
- 4-(3-Aminopropyl)morpholine to yield 58p

EXAMPLE 17

Synthesis Of Compound 64 And Its Related Compounds

The reaction scheme for the general synthesis of compound 64 and its precursors is given in FIGURE 23.

2-Methylbenzoxazole, 59, is obtained from the Aldrich Chemical Company (Milwaukee, WI). It is alkylated to produce compound 60 using
30 methyl iodide by adapting procedures such as found in Southwick et al. US

Patent No. 4,981,977, Jan. 1, 1991 or in Ernst et al., Cytometry, 10, 1989, pp. 3-10. Compound 45 is obtained from the Aldrich Chemical Company and is reacted with 3-bromo-1-propanol (also available from the Aldrich Chemical Company) by adapting procedures Gaugain, et. al, Biochemistry, 5 Vol. 17, No. 24, 1978, pp. 5071-5078. The condensation of compound 60 and 46 is effected by adapting procedures found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and Related Compounds", Wiley, 1964, pg. 37 to yield compound 61. Compound 62 can then be obtained by converting the alcohol to the tosylate by using procedures such as found in 10 Wiberg, K. et al., J. Am. Chem. Soc., 92 (3), 1970, pp. 553-564. The tosylate 62 is then converted to the bromide via a nucleophilic displacement reaction with sodium bromide as described by Wilt, J., J. Org. Chem., 35 (8), 1970, pp. 2803-2806 to yield compound 63.

Alternatively, a one step procedure is provided in Hooz, J. et al., 15 Can. J. Chem., 46, 1968, pp. 86-87.

Compound 63 can then be reacted with diethylene triamine (available from the Aldrich Chemical company) by the following procedure.

Compound 63 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 20 ml) is then added to this flask while stirring under nitrogen and the diethylene triamine (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. 25 Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon 30 cooling to 0°C for 15 minutes. A second crop is also collected from the

second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 64 is obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Amine derivatives of compound 63 can be synthesized as follows.

Compound 63 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the appropriate amine selected from the following list (0.283 moles), which are available from the Aldrich Chemical Company, is added while stirring is continued.

The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 30a-30o is obtained and is subjected to high vacuum overnight.

Listed below are the compounds that can be obtained from their corresponding amines.

4-amino-1-benzylpiperidine to yield 63a
spermine to yield to yield 63b
pyridine to yield to yield 63c

- 2-(2-Aminoethyl)-1-methylpyrrolidine to yield 63d
1-(2-Aminoethyl)pyrrolidine to yield 63e
1-(2-Aminoethyl)piperidine to yield 63f
2-(2-Aminoethyl)pyridine to yield 63g
5 1-(2-Aminoethyl)piperazine to yield 63h
4-(2-Aminoethyl)morpholine to yield 63i
1-Amino4-(2-hydroxyethyl)piperazine to yield 63j
4-(Aminomethyl)piperidine to yield 63k
2-(Aminomethyl)pyridine to yield 63l
10 aniline to yield 63m
1-(3-Aminopropyl)imidazole to yield 63n
4-(3-Aminopropyl)morpholine to yield 63o

Characterization can be effected by calculating the molecular mass of
15 the free base amine from the exact isotopic mass formulas well known to
those skilled in the art and comparing the resultant mass with that obtained
by a high resolution mass spectrometry molecular weight determination such
as are well known to those skilled in the art. Note that no hydrolysis is
necessary.

20 Since the structures of compounds 63a-63o are unambiguous from the
above generic procedures, their structures are not shown.

EXAMPLE 18

25 Synthesis Of Compounds 68 And 68a-68p

The reaction scheme for the general synthesis of compounds 68, 68a,
and their precursors, is given in FIGURE 24. The structure formula of
compounds 68a-68p are given in FIGURE 25.

2-Methylbenzoxazole 59 is obtained from the Aldrich Chemical
30 Company (Milwaukee, WI). It is alkylated to produce compound 65 using

3-brom-1-propanol by adapting procedures of Gaugain et al., Biochemistry, 17 (24), 1978, pp. 5071-5078. Compound 45 is obtained from the Aldrich Chemical Company and is reacted with 3-bromo-1-propanol (also available from the Aldrich Chemical Company) using procedures such as found in
5 Gaugain et al., Biochemistry, 17 (24) 1978, 5071-5078. The condensation of compound 65 and 46 is effected by adapting procedures found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and Related Compounds", Wiley, 1964, p. 37 to yield compound 60. Compound 67 is then obtained by converting the alcohol to the tosylate by using procedures
10 such as found in Wiberg, K. et al., J. Am. Chem. Soc., 92 (3), 1970, pp. 553-564. The tosylate 67 is then converted to the bromide via a nucleophilic displacement reaction with sodium bromide as per Wilt, J., J. Org. Chem., 35 (8), 1970, pp. 2803-2806 to yield compound 68.

Alternatively, a one step procedure, as provided by Hooz, J. et al.,
15 Can. J. Chem., 46, 1968, pp. 86-87, can be used.

Compound 68 can then be reacted with diethylene triamine or other appropriate amine (available from the Aldrich Chemical Company) by the following procedure.

Compound 68 (0.0081 moles) is added to a 250 ml round bottom
20 flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and an amine, such as diethylene triamine or other appropriate base (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under
25 nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with
30 10 N HCl. After filtration of this suspension, the resultant solid is

recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 68a is obtained and is subjected to high vacuum overnight. Characterization is effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

As long as the ratios of reagents and solvents are held constant, one can scale up or down the amounts of reagents using the method of ratio and proportions well known to those skilled in the art.

Listed below are products that can be obtained from their corresponding amines.

- diethylene triamine to yield compound 68a
- 4-amino-1-benzylpiperidine to yield compound 68b
- spermine to yield compound 68c
- pyridine to yield compound 68d
- 2-(2-Aminoethyl)-1-methylpyrrolidine to yield compound 68e
- 1-(2-Aminoethyl)pyrrolidine to yield compound 68f
- 1-(2-Aminoethyl)piperidine to yield compound 68g
- 2-(2-Aminoethyl)pyridine to yield compound 68h
- 1-(2-Aminoethyl)piperazine to yield compound 68i
- 4-(2-Aminoethyl)morpholine to yield compound 68j
- 1-Amino-4-(2-hydroxyethyl)piperazine to yield 68k
- 4-(Aminomethyl)piperidine to yield 68l
- 2-(Aminomethyl)pyridine to yield 68m
- aniline to yield 68n
- 1-(3-Aminopropyl)imidazole to yield 68o
- 4-(3-Aminopropyl)morpholine to yield 68p

EXAMPLE 19

Synthesis Of Compounds 71 And 71a-71p

The reaction scheme for the general synthesis of compounds 71, 71a, and their precursors, is given in FIGURE 26. The structure formula of compounds 71a-71p are given in FIGURE 27.

2-Methylbenzoxazole 59 is obtained from the Aldrich Chemical Company (Milwaukee, WI). It is alkylated to produce compound 65 using 3-bromo-1-propanol by adapting procedures such as found in Gaugain, et. al, Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078 to obtain compound 13. Compound 45 is obtained from the Aldrich Chemical Company and is reacted with methyl iodide (also available from the Aldrich Chemical Company) using procedures such as found in Southwick et al. US Patent No. 4,981,977, Jan. 1, 1991 or in Ernst et al., Cytometry, 10, 1989, pp. 3-10 to yield compound 55. The condensation of compound 65 and 55 is effected by adapting procedures found in Hamer, Francis, "Heterocyclic Compounds, Cyanine Dyes and Related Compounds", Wiley, 1964, p. 37 to yield compound 69. Compound 70 is then obtained by converting the alcohol to the tosylate by using procedures such as found in Wiberg, K. et al., J. Am. Chem. Soc., 92 (3), 1970, pp. 553-564. The tosylate 70 can then be converted to the bromide via a nucleophilic displacement reaction with sodium bromide as disclosed by Wilt, J., J. Org. Chem., 35 (8), 1970, pp. 2803-2806 to yield compound 20.

Alternatively, a one step procedure, as provided by Hooz, J. et al., Can. J. Chem., 46, 1968, pp. 86-87, can be used. Compound 70 can then be reacted with diethylene triamine or other appropriate amine (available from the Aldrich Chemical company) by the following procedure.

Compound 71 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and diethylene triamine or other appropriate amine, (0.283 moles), which is available from

the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the final product 71a-71p is obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

As long as the ratios of reagents and solvents are held constant, one can scale up or down the amounts of reagents using the method of ratio and proportions well known to those skilled in the art.

Listed below are products that can be obtained for their corresponding amines.

- diethylene triamine to yield compound 20a
- 4-amino-1-benzylpiperidine to yield compound 20b
- spermine to yield compound 20c
- pyridine to yield compound 20d
- 2-(2-Aminoethyl)-1-methylpyrrolidine to yield compound 20e
- 1-(2-Aminoethyl)pyrrolidine to yield compound 20f
- 1-(2-Aminoethyl)piperidine to yield compound 20g
- 2-(2-Aminoethyl)pyridine to yield compound 20h

1-(2-Aminoethyl)piperazine to yield compound 20i
4-(2-Aminoethyl)morpholine to yield compound 20j
1-Amino4-(2-hydroxyethyl)piperazine to yield 20k
4-(Aminomethyl)piperidine to yield 20l
5 2-(Aminomethyl)pyridine to yield 20m
aniline to yield 20n
1-(3-Aminopropyl)imidazole to yield 20o
4-(3-Aminopropyl)morpholine to yield 20p

10

EXAMPLE 20

Synthesis Of Compound 80 And Its Related Compounds

The reaction scheme for the general synthesis of compound 80 and its precursors is given in FIGURE 28.

15

Compound 72 is obtained from the Aldrich Chemical Company. Compound 73 can be synthesized from compound 72, compound 74 can be synthesized from compound 73, and compound 75 can be synthesized from compound 74, each following the procedure of Dervan et al., J. Am. Chem. Soc., Vol. 100, No. 6, 1978, pp. 1968-1970 or secondary references
20 contained within.

Compound 76 is synthesized from compound 75 by the procedure of Gaugain, et. al, Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078.

Compound 77 can be synthesized from compound 76 by the procedure of Dervan, P.B. , Becker, M.M., J. Am. Chem. Soc., 1978, Vol. 100, No.

25

6, 1968-1970 or secondary references contained within. Compound 78 can be synthesized from compound 77 by the procedures such as Lee et al., J. Am. Chem. Soc., 88 (14), 1966, pp. 3440-3441 or references contained within. Compound 79 can be synthesized by the following procedure.

Compound 78 (0.0081 moles) is added to a 250 ml round bottom flask
30 equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml)

is then added to this flask while stirring under nitrogen and the diethylene triamine (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 79 is obtained and is subjected to high vacuum overnight. Characterization is effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art. The final compound 80 can be obtained by using the reduction procedure of Dervan, P.B. , Becker, M.M., J. Am. Chem. Soc., 1978, 100 (6), 1968-1970. Characterization and purification of all intermediates can be accomplished using methods well known to those skilled in the art.

Amine derivatives of compound 78 can be synthesized as follows. Compound 78 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condensor. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the appropriate amine selected from the following list (0.283 moles), which are available from the Aldrich Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto

- distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N
- 5 HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 78a-78o is obtained and is subjected to high vacuum overnight.
- 10 Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.
- 15 Given below are the compounds that can be obtained from their corresponding amines.
- 4-amino-1-benzylpiperidine to yield 78a
spermine to yield to yield 78b
pyridine to yield to yield 78c
- 20 2-(2-Aminoethyl)-1-methylpyrrolidine to yield 78d
1-(2-Aminoethyl)pyrrolidine to yield 78e
1-(2-Aminoethyl)piperidine to yield 78f
2-(2-Aminoethyl)pyridine to yield 78g
1-(2-Aminoethyl)piperazine to yield 78h
- 25 4-(2-Aminoethyl)morpholine to yield 78i
1-Amino4-(2-hydroxyethyl)piperazine to yield 78j
4-(Aminomethyl)piperidine to yield 78k
2-(Aminomethyl)pyridine to yield 78l
aniline to yield 78m
- 30 1-(3-Aminopropyl)imidazole to yield 78n

4-(3-Aminopropyl)morpholine to yield 78o

Synthesis and purification of the final product 78aa-78oo can be accomplished by the following protocol to yield the corresponding final products as shown below. The nitro compound 78a-78o can be converted to the appropriate amine using the procedure of Dervan et al., J. Am. Chem. Soc., 100 (6), 1978, pp. 1968-1970.

Listed below are the products that can be obtained from their corresponding immediate precursors.

10 78a to yield 78aa
 78b to yield to yield 78bb
 78c to yield to yield 78cc
 78d to yield 78dd
 78e to yield 78ee
15 78f to yield 78ff
 78g to yield 78gg
 78h to yield 78hh
 78i to yield 78ii
 78j to yield 78jj
20 78k to yield 78kk
 78l to yield 78ll
 78m to yield 78mm
 78n to yield 78nn
 78o to yield 78oo

25

Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

30

Since the structures of compounds 78a-78o and 78aa-78oo are unambiguous from the given generic procedures, their structures are not shown.

5

EXAMPLE 21

Synthesis Of Compound 85 And Its Related Compounds

The reaction scheme for the general synthesis of compound 85 and its precursors is given in FIGURE 29.

10 Starting material 81 (0.0876 moles) is obtained from the Aldrich Chemical Company (Milwaukee, WI) and is added to a single neck 3.0 liter round bottom flask under Argon and equipped with a magnetic stir bar and a reflux condenser. To this vessel, 1.0 liter of dry pyridine is added while stirring. Stirring of the resulting suspension is continued for 15 minutes
15 until all the solid is dissolved. A catalytic amount of N,N-dimethylaminopyridine (1.07 g, 0.00876 moles) is added to this solution while stirring. Acetic anhydride (462 g, 4.9 moles) is then added and the resulting reaction mixture is refluxed for 8-12 hours. The reaction mixture is then allowed to cool and the solvent is removed in vacuo. For
20 purification of the product 42, a gradient silica gel column is performed using an appropriate solvent system determined using methods well known to those skilled in the art such as Thin Layer Chromatography. Fractions of 10.0 ml are collected and appropriate fractions are recombined and the solvent is removed in vacuo. The residue is then dissolved in hot EtOH
25 (220 ml) and precipitated by cooling to 0°C. The mother liquor is decanted off and 200 ml of fresh EtOH is added. The solid is redissolved by heating and is allowed to crystallize and -4°C for 48 hours. Crystals are collected from both the mother liquor and the second recrystallization and are washed with a small amount of cold EtOH and dried under high vacuum for several

hours. The resultant compound 82 can be characterized by high resolution mass spectrometry as well known to those skilled in the art.

Compound 83 can be synthesized from the imide 42 via a modification of a literature procedure of Gaugain, et al., Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078 for quarternization of the diamide of 3,8 diamino-6-phenyl phenanthridine. Imide 82 (0.023 moles) is placed in a 2.0 liter round bottom flask under Argon and is equipped with a magnetic stir bar and reflux condenser. 1,3-Dibromopropane (1.0 liter, 9.86 moles) is added to this flask and the resultant mixture is brought to reflux for about 7 hours. The solution is cooled overnight and the precipitant is filtered and washed with Et₂O. This material is recrystallized from CH₃OH to yield diacetyl bromide 83 which can be characterized by mass spectrometry and other methods known to those skilled in the art.

Compound 83 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the diethylene triamine (0.283 moles), which is available from the Aldrich Chemical Company is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for about 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 84 can be obtained and can be subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the free base amine

from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

5 Synthesis and purification of the final product 85 can be accomplished by the following protocol. The imide (0.0036 moles) 84 is dissolved in 75.0 ml methanol and 75 ml of 4N HCl is added. The mixture is refluxed for about 2 hours and allowed to cool. Ethanol is added to this solution and resulting precipitate is filtered and washed with a minimal
10 amount of cold ethanol. The filtrate is reconcentrated and fresh ethanol and concentrated aqueous HCl is added. This resulting precipitate is also filtered. Next, this filtrate is concentrated to near dryness and Et₂O is added and the solid filtered off. The last remaining unfilterable residue is then dissolved in concentrated HCl and precipitated with EtOH. This material is
15 filtered and washed with Ethanol. All solid materials are combined from the above sequence and is subjected to high vacuum overnight to obtain 85. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a
20 high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

 Amine derivatives of compound 83 can be synthesized as follows: Compound 83 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml)
25 is then added to this flask while stirring under nitrogen and the appropriate amine selected from the following list (0.283 moles), which are available from the Aldrich Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto
30 distilled H₂O. Then, this mixture is concentrated in vacuo until only the

H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized
5 from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 83a-83o can be obtained and is subjected to high vacuum overnight. Characterization can be effected by calculating the molecular mass of the
10 free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Listed below are the products that can be obtained from their
15 corresponding amines.

- 4-amino-1-benzylpiperidine to yield 83a
- spermine to yield to yield 83b
- pyridine to yield to yield 83c
- 2-(2-Aminoethyl)-1-methylpyrrolidine to yield 83d
- 20 1-(2-Aminoethyl)pyrrolidine to yield 83e
- 1-(2-Aminoethyl)piperidine to yield 83f
- 2-(2Aminoethyl)pyridine to yield 83g
- 1-(2-Aminoethyl)piperazine to yield 83h
- 4-(2-Aminoethyl)morpholine to yield 83i
- 25 1-Amino4-(2-hydroxyethyl)piperazine to yield 83j
- 4-(Aminomethyl)piperidine to yield 83k
- 2-(Aminomethyl)pyridine to yield 83l
- aniline to yield 83m
- 1-(3-Aminopropyl)imidazole to yield 83n
- 30 4-(3-Aminopropyl)morpholine to yield 83o

Synthesis and purification of the final product 83aa-83oo can be accomplished by the following protocol to yield the corresponding final products as shown below. The imide (0.0036 moles) 83a-83o is dissolved in 75.0 ml methanol and 75 ml of 4N HCl is added. The mixture is
5 refluxed for about 2 hours and allowed to cool. Ethanol is added to this solution and resulting precipitate is filtered and washed with a minimal amount of cold ethanol. The filtrate is reconcentrated and fresh ethanol and concentrated aqueous HCl is added. This resulting precipitate is also filtered. Next, this filtrate is concentrated to near dryness and Et₂O is added
10 and the solid filtered off. The last remaining unfilterable residue is then dissolved in concentrated HCl and precipitated with EtOH. This material is filtered and washed with Ethanol. All solid materials are combined from the above sequence and is subjected to high vacuum overnight to obtain 83aa-83oo. Characterization can be effected by calculating the molecular mass of
15 the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Listed below are the products that can be obtained from their
20 corresponding immediate starting materials.

83a to yield 83aa
83b to yield 83bb
83c to yield 83cc
83d to yield 83dd
25 83e to yield 83ee
83f to yield 83ff
83g to yield 83gg
83h to yield 83hh
83i to yield 83ii
30 83j to yield 83jj

83k to yield 83kk

83l to yield 83ll

83m to yield 83mm

83n to yield 83nn

5 83o to yield 83oo

Since the structures of compounds 83a-83o and 83aa-83oo are unambiguous from the generic procedures, their structures are not given.

10

EXAMPLE 22

Synthesis Of Compound 90 And Its Related Compounds

The reaction scheme for the general synthesis of compound 90 and its precursors is given in FIGURE 30.

15 Starting material 86 (0.0876 moles) is obtained from the Aldrich Chemical Company (Milwaukee, WI) and is added to a single neck 3.0 liter round bottom flask under Argon and equipped with a magnetic stir bar and a reflux condenser. Acetic anhydride (462 g, 4.9 moles) is then added and the resulting reaction mixture is refluxed for 8-12 hours. The reaction
20 mixture is then allowed to cool and the solvent is removed in vacuo. For purification of the product 87, a gradient silica gel column is performed using an appropriate solvent system determined using methods well known to those skilled in the art. Fractions of 10.0 ml are collected and appropriate fractions are recombined and the solvent is removed in vacuo.
25 The residue is then dissolved in hot EtOH (220 ml) and precipitated by cooling to 0°C. The mother liquor is decanted off and 200 ml of fresh EtOH is added. The solid is redissolved by heating and is allowed to crystallize and -4°C for 48 hours. Crystals are collected from both the mother liquor and the second recrystallization and are washed with a small
30 amount of cold EtOH and dried under high vacuum for several hours. The

resultant compound 87 can be characterized by high resolution mass spectrometry as well known to those skilled in the art.

Compound 88 can be synthesized from the diamide 87 via a modification of a literature procedure of Gaugain, et al., Biochemistry, Vol. 17, No. 24, 1978, pp. 5071-5078 for quaternization of the diamide of 3,8-diamino-6-phenyl phenanthridine. Diamide 87 (0.023 moles) is placed in a 2.0 liter round bottom flask under Argon and is equipped with a magnetic stir bar and reflux condenser. 1,3-Dibromopropane (1.0 liter, 9.86 moles) is added to this flask and the resultant mixture is brought to reflux for about 7 hours. The solution is cooled overnight and the precipitant is filtered and washed with Et₂O. This material can be recrystallized from CH₃OH to yield diacetyl bromide 88 which can be characterized by mass spectrometry and other methods known to those skilled in the art.

Compound 88 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml) is then added to this flask while stirring under nitrogen and the diethylene triamine (0.283 moles), which is available from the Aldrich Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution is then allowed to cool to room temperature and is poured onto distilled H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl. After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the second filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 89 can be obtained and is subjected to high vacuum overnight. Characterization can be

5 effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Synthesis and purification of the final product 90 can be accomplished by the following protocol. The diamide (0.0036 moles) 89 is dissolved in 75.0 ml methanol and 75 ml of 4N HCl is added. The mixture is refluxed for 2 hours and allowed to cool. Ethanol is added to this
10 solution and resulting precipitate is filtered and washed with a minimal amount of cold ethanol. The filtrate is reconcentrated and fresh ethanol and concentrated aqueous HCl is added. This resulting precipitate is also filtered. Next, this filtrate is concentrated to near dryness and Et₂O is added and the solid filtered off. The last remaining unfilterable residue is then
15 dissolved in concentrated HCl and precipitated with EtOH. This material is filtered and washed with Ethanol. All solid materials are combined from the above sequence and subjected to high vacuum overnight to obtain compound 90. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well
20 known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Amine derivatives of compound 88 can be synthesized as follows: Compound 88 (0.0081 moles) is added to a 250 ml round bottom flask equipped with a magnetic stir bar and reflux condenser. Methanol (150 ml)
25 is then added to this flask while stirring under nitrogen and the appropriate amine selected from the following list (0.283 moles), which are available from the Chemical Company, is added while stirring is continued. The resultant solution is heated to reflux overnight under nitrogen. This solution
30 is then allowed to cool to room temperature and is poured onto distilled

H₂O. Then, this mixture is concentrated in vacuo until only the H₂O remains. An additional 50-75 ml H₂O is added and the reaction mixture is cooled to 0°C. The solid is filtered and washed with ice cold water. This material is then redissolved in EtOH and precipitated with 10 N HCl.

5 After filtration of this suspension, the resultant solid is recrystallized from hot ethanol upon cooling to 0°C for 15 minutes. A second crop is also collected from the 2nd filtrate upon standing and by precipitation with EtOH from the first filtrate. These solids are then combined and the product 48a-48o is obtained and is subjected to high vacuum overnight. Characterization

10 can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

15 By combining the generic procedure for the synthesis of phenanthridinium derivatives 28a-42a and 28b-42b with the above procedures for the synthesis of bromide compound 88, 83, 78, 63 or 49, any combination of amine tail - intercalator molecular segments using the generic procedure for alkylation of bromides with amines as already

20 described above can be synthesized, except that for derivatives of compounds 49 and 63, no hydrolysis step is need so the hydrolysis step is eliminated. In the case of derivatives of compound 78 no hydrolysis step will be needed but a reduction step is needed according to the procedure of Dervan et al., J. Am. Chem. Soc., 100 (6), 1978, pp. 1968-1970.

25

EXAMPLE 23

General Procedure To Form A "Matrix" Of Compounds

Synthesis and purification of the final product 88aa-88oo can be

30 accomplished by the following protocol to yield the corresponding final

products as shown below. The imide (0.0036 moles) 88a-88o is dissolved in 75.0 ml methanol and 75 ml of 4N HCl is added. The mixture is refluxed for 2 hours and allowed to cool. Ethanol is added to this solution and resulting precipitate is filtered and washed with a minimal amount of cold ethanol. The filtrate is reconcentrated and fresh ethanol and concentrated aqueous HCl is added. This resulting precipitate is also filtered. Next, this filtrate is concentrated to near dryness and Et₂O is added and the solid filtered off. The last remaining unfilterable residue is then dissolved in concentrated HCl and precipitated with EtOH. This material is filtered and washed with Ethanol. All solid materials are combined from the above sequence and is subjected to high vacuum overnight to obtain 88aa-88oo. Characterization can be effected by calculating the molecular mass of the free base amine from the exact isotopic mass formulas well known to those skilled in the art and comparing the resultant mass with that obtained by a high resolution mass spectrometry molecular weight determination such as are well known to those skilled in the art.

Listed below are the products that can be obtained from their corresponding immediate precursors.

	<u>88a</u> to yield <u>88aa</u>
20	<u>88b</u> to yield <u>88bb</u>
	<u>88c</u> to yield <u>88cc</u>
	<u>88d</u> to yield <u>88dd</u>
	<u>88e</u> to yield <u>88ee</u>
	<u>88f</u> to yield <u>88ff</u>
25	<u>88g</u> to yield <u>88gg</u>
	<u>88h</u> to yield <u>88hh</u>
	<u>88i</u> to yield <u>88ii</u>
	<u>88j</u> to yield <u>88jj</u>
	<u>88k</u> to yield <u>88kk</u>
30	<u>88l</u> to yield <u>88ll</u>

88m to yield 88mm

88n to yield 88nn

88o to yield 88oo

- 5 Since the structures of compounds 88a-88o and 88aa-88oo are unambiguous from the generic procedures, their structures are not shown.

EXAMPLE 24

10 Hybridization Assay Using Compounds 24 - 27

- In four separate experiments, PTA 24, compound 25, compound 26, and compound 27 were used to quantitate hybridization when a target oligonucleotide was titrated with its complementary partner. By also conducting three simultaneous but independent experiments, a comparison of
- 15 ethidium bromide staining, propidium iodide staining and ethidium homodimer staining versus PTA 24 was made as follows. Results can be found in FIGURE 31. Results of a comparison of ethidium bromide staining, compound 24 (PTA) staining, compound 25 staining, compound 26 staining and compound 27 staining, as per the following protocol, are found
- 20 in FIGURE 32. Complementary strands of DNA oligodeoxythymidylic acid, d(pT)_n, and oligodeoxyadenylic acid, (d(pA)_n), were obtained from the Sigma Chemical Co. in St. Louis, MO. A stock solution of d(pA)_n was made at 5.0 units/0.5 ml of 0.004 M TRIS, 0.001 M EDTA, pH 8.2 buffer. For polyA, $\epsilon = 8.4$ AU/mM cm or $8400 \text{ M}^{-1}\text{cm}^{-1}$; Therefore, with 9 base
- 25 pairs for d(pA)₉, the ϵ is $75,600 \text{ M}^{-1}\text{cm}^{-1}$. This stock was then diluted 100X to obtain stock at $6.61 \times 10^{-7} \text{ M}$, or $0.66 \mu\text{M}$. The d(pT)₉ stock was made at 25 units/5.0 ml and used for titration after dilution 10X with the same buffer. Since the ϵ for polyT is 8.15 AU/mM cm or $8,150 \text{ M}^{-1}\text{cm}^{-1}$ per base pair, or $73,350 \text{ M}^{-1}\text{cm}^{-1}$ per oligo, the concentration of the oligo stock was
- 30 $6.8 \mu\text{M}$ in DNA molecules. A titration was performed using a Hitachi F-

4010 Fluorescence Spectrophotometer using polystyrene disposable 4.0 ml cuvettes to obtain a fully corrected spectra and an excitation wavelength of 488-550 nm (using 488 nm for this experiment) and an emission wavelength of 600-650 nm (using 625 nm for this experiment). Equivalents of d(pT),
5 were added at the following increments: 0.020, 0.080, 0.150, 0.300, 0.500, 0.700, 1.000, 2.000, 5.000 equivalents. Each sample in the titration curve was prepared individually by dividing the initial d(TA), stock in 10 x 1.0 ml increments. The addition of complement was then accomplished by micropipetting an appropriate amount (2, 5, 8, 15, 30, 50, 70, 100, 200,
10 and 500 μ l, respectively) of d(pT)9 stock to each of a series of the 10 aliquots. Each aliquot, containing progressively larger molar ratios of the two complementary strands, was incubated at ambient temperature for 1 hr 45 minutes, the corresponding dye was added as 100.0 μ l aliquots of a 15 μ M solution of the corresponding dye in 0.004M TRIS, 0.001M EDTA, pH
15 8.2 buffer. This corresponds to a dye/DNA b.p. ratio of 1/4 at saturation with complementary oligo. Overall concentrations of dye and oligo vary in the saturation plot because of the use of varied increments additions from the same stock solution. After an additional 45 minutes incubation time after addition of the corresponding appropriate dye, the relative fluorescence
20 intensity was read at 625 nm and recorded to generate a standard curve which is directly proportional to the quantity of dsDNA hybridization, or target sequence, under the same conditions. The background, or initial residual fluorescence, is then substrated out as a constant for all curves for comparison of the various titration curves on the same graphs.

25 It is apparent to those skilled in the art that the high binding affinity of the intercalator compounds 24, 25, 26, or 27 of the present invention is especially useful relative to conventional intercalators such as ethidium bromide (FIGURE 32). In FIGURE 32 (EXAMPLE 24), the sensitivity or response to ds-DNA concentration in the presence of intercalator compounds
30 24, 25, 26, and 27 is shown compared to ethidium bromide. When

concentrations of oligonucleotide are less than 10^{-6} M, the advantages of such high affinity intercalators is especially apparent.

In comparison of PTA 24 to homobifunctional intercalators such as ethidium homodimer (FIGURE 32), again a clear advantage is sensitivity is
5 observed. In this case, the increased sensitivity may be related to reduced self-quenching in the PTA 24 as well as its high affinity for DNA.

In FIGURE 8 and FIGURE 9, the concentration of ds-DNA was higher and the differential between the ethidium bromide and PTA 24 (FIGURE 9) and ethidium homodimer and PTA 24 (FIGURE 8) was not
10 significant because of the higher concentration of DNA used.

In summary, the present invention offers clear advantages in allowing the detection of ds-DNA hybridization at much lower concentrations than conventional staining methods currently available or known in the art.

15

EXAMPLE 25

Hybridization Assay /Using Intercalator Compound 1, 2, 3, 24, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80

20 DNA Intercalator 1, 2, 3, 24, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80 can be used to quantitate hybridization when a target oligonucleotide is titrated with its complementary partner. Complementary strands of DNA oligodeoxythymidylic acid, d(pT)_n, and oligodeoxyadenylic acid, (d(pA)_n),
25 can be obtained from the Sigma Chemical Co. in St. Louis, MO. A stock solution of d(pA)₉ is made at 5 units/ml of 0.05M TRIS, 0.2N NaCl, 1mM EDTA, pH 8.4 or other suitable buffer. For polyA, $\epsilon = 8.4$ AU/mM cm or $8,400 \text{ M}^{-1}\text{cm}^{-1}$; therefore, with 9 base pairs for d(pA)₉, the ϵ is $75,600 \text{ M}^{-1}\text{cm}^{-1}$. This stock is then diluted to obtain stock from 0.066 - 66 μM . The
30 d(pT)₉ stock is made at 25 units/5.0 ml and used for titration without further dilution in the same buffer. Since the ϵ for polyT is 8.15 AU/mM cm or

8,150 M-1cm⁻¹ per base pair, or 73,350 M-1cm⁻¹ per oligo, the concentration of the oligo stock is 0.0068 - 660 μ M in DNA molecules. A titration can be performed using a Fluorescence Spectrophotometer using an excitation wavelength of 488-550 nm (optimal around 534) and an emission wavelength of 600-650 nm (optimal around 625). Equivalents of d(pT)₉ is added at the following increments: 0.02, 0.05, 0.080, 0.150, 0.300, 0.500, 0.700, 1.00, 2.00, 5.00 equivalents. Each sample in the titration curve is prepared individually by dividing the initial d(pA)₉ stock into 10 x 1.0 ml aliquots. The addition of complement is then accomplished by micropipetting an appropriate amount (2, 5, 8, 15, 30, 50, 70, 100, 200, and 500 μ l, respectively) of d(pT)₉ stock to each of a series of the 10 aliquots when the d(pT)₉ stock is 10X the concentration of the d(pA)₉ stock. Each aliquot, obtaining progressively larger molar ratios of the two complementary strands, is incubated at ambient temperature for 0.25 - 2.5 hours, the dye is added as 1-500 μ l aliquots of a 1- 1000 μ M solution of the dye in 0.05M TRIS, 0.2N NaCl, 1mM EDTA, pH 8.4 buffer or other suitable buffer. This corresponds to a dye/DNA b.p. ratio of 1/1 - 1/1000 at saturation with complementary oligo. Overall concentrations of dye and oligo vary in the saturation plot because of the use of varied increments additions from the same stock solution. After an additional 15 minute incubation time, the relative fluorescence intensity is then read at between 580 - 680 nm and recorded to generate a standard curve which is directly proportional to the quantity of dsDNA hybridization, or target sequence, under these conditions.

25

EXAMPLE 26

Determination of Hybridization of Complementary Binding Pairs
Other Than d(pT)9 and d(dA)9 Using Intercalator Compound 1, 2, 3,
24, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b,
38, 39b, 40b, 41b, 42b, or 80

By using the above described procedure from EXAMPLE 25,
intercalator compound 1, 2, 3, 24, 25, 26, 27, 28b, 29b, 30b, 31b, 32b,
33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80 can be used to
determine the quantity of hybridization of any other complementary DNA
strands by substituting d(pT)9 with the appropriate complementary DNA and
substituting d(pA)9 with the appropriate target DNA at appropriate
concentrations that are determined by one skilled in the art and depending
on the degree of complementary regions that are expected as is determined
by one skilled in the art. In each case, the excitation wavelength of 450-550
nm (optimal at 534 nm) can be used and the relative fluorescence intensity is
then read at between 580 - 680 nm and recorded to generate a standard
curve which is directly proportional to the quantity of dsDNA hybridization,
or target sequence, under these conditions.

EXAMPLE 27

Hybridization Assay using Intercalator Compound 7, 8, 50, 54a, 54b, 54c,
54d, 54e, 54f, 54g, 54h, 54i, 54j, 54k, 54l, 54m, 54n, 54o, 54p,
58a, 58b, 58c, 58d, 58e, 58f, 58g, 58h, 58i, 58j, 58k, 58l, 58m,
58n, 58o, 58p, 64, 68a, 68b, 68c, 68d, 68e, 68f, 68g, 68h, 68i,
68j, 68k, 68l, 68m, 68n, 68o, 68p, 71a, 71b, 71c, 71d, 71e, 71f,
71g, 71h, 71i, 71j, 71k, 71l, 71m, 71n, 71o, or 71p

By using the above procedure in EXAMPLE 26, the hybridization of
any two complementary strands can be determined using intercalator
compound 7, 8, 50, 54a, 54b, 54c, 54d, 54e, 54f, 54g, 54h, 54i, 54j, 54k,
54l, 54m, 54n, 54o, 54p, 58a, 58b, 58c, 58d, 58e, 58f, 58g, 58h, 58i, 58j,
58k, 58l, 58m, 58n, 58o, 58p, 64, 68a, 68b, 68c, 68d, 68e, 68f, 68g, 68h,
68i, 68j, 68k, 68l, 68m, 68n, 68o, 68p, 71a, 71b, 71c, 71d, 71e, 71f, 71g,

71h, 71i, 71j, 71k, 71l, 71m, 71n, 71o, or 71p as described in EXAMPLE 26 except the wavelengths of excitation and emission are optimized as determined by one skilled in the art before conducting the assay. In each case, the relative fluorescence intensity is then read and recorded to generate a standard curve which is directly proportional to the quantity of dsDNA hybridization, or target sequence, under these conditions.

EXAMPLE 28

Hybridization Assay for d(pT)9 and d(pA)9 Using Intercalator Compound 7, 8, 50, 54a, 54b, 54c, 54d, 54e, 54f, 54g, 54h, 54i, 54j, 54k, 54l, 54m, 54n, 54o, 54p, 58a, 58b, 58c, 58d, 58e, 58f, 58g, 58h, 58i, 58j, 58k, 58l, 58m, 58n, 58o, 58p, 64, 68a, 68b, 68c, 68d, 68e, 68f, 68g, 68h, 68i, 68j, 68k, 68l, 68m, 68n, 68o, 68p, 71a, 71b, 71c, 71d, 71e, 71f, 71g, 71h, 71i, 71j, 71k, 71l, 71m, 71n, 71o, or 71p

By using the above described procedure in EXAMPLE 25, complementary oligonucleotide d(pA)9 and d(pT)9 hybridization can be determined except that the wavelengths are substituted to optimal wavelengths for each compound as is determined by one skilled in the art. The described procedure is then followed using the optimal wavelengths as are determined.

EXAMPLE 29

Gel Electrophoresis Application Using Intercalator Compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38b, 39b, 40b, 41b, 42b, or 80

An agarose gel can be run to detect DNA using compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38b, 39b, 40b, 41b, 42b, or 80. Plasmid, pBR322, at 2.1 mg in 7 ml stock is incubated at 37°C for 1 hour with 1 ml of BAMH restriction enzyme with 2 ml 10X React2 Buffer and diluted to 20 ml total with 10 ml H₂O. Alternatively, any other appropriate DNA sample is substituted for the

above described "nicked" plasmid. This mixture is then used to prepare 3 stocks of nicked pBR322 plasmid at 0.63 mg per 6 ml for each vial. Each of these stocks is diluted further with H₂O and 20% glycerol to final DNA stocks of 20 ng/ml, 800 pg/ml, 160 pg/ml, and 40 pg/ml with a 1:4 ratio of dye to DNA base pairs in each for a total of 12 stocks. A 5 μ l aliquot of each stock is loaded into 12 separate lanes in agarose gel and electrophoresis is run for 30 minutes in 4 mM TRIS, pH8.2, with 0.01mM EDTA buffer. The gel is then removed and photographed under exposure to U.V. light in a conventional gel box. Alternatively, the gel is scanned using fluorescence confocal microscopy or charge coupled device imaging as a fluorescence detection or visualization method.

EXAMPLE 30

15 Protocol for Synthesis of Intercalator Activated Carboxymethyl
Styrene Microparticle Capture Reagent Using Intercalator
Compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64,
68a, 68c, 71a, 71c, 80, 85, or 90

20 The synthesis of intercalator derivatized solid phase microparticle (MP) capture reagent can be accomplished by the following procedure:

25 A 45 aliquot of $0.275 \pm \mu$ m microparticles (Seradyne, Indianapolis, IN) is placed in a 4 ml vial and the surfactant is exchanged out using Bio-Rex 501-D ion exchange mixed bed resin (Bio-Rad, Richmond, CA). After gentle shaking for 2 hours, the resin is filtered out from the mixture by
30 using a coarse fritted glass funnel equipped with a reduced pressure collection chamber. The sample is diluted to a concentration of mp at 10% solids by weight. The total amount of equivalents of reactive carboxylic acid is calculated from the titration specifications of the vendor. A stock solution of sulfo N-hydroxysuccinimide (Pierce, Rockford, IL) is made at
30 11 mg/ml (20 mM) in H₂O and a stock solution of EDAC (Sigma Chemical Co., St. Louis, MO) at 10 mg/ml (5 mM) is made in H₂O. Five

equivalents of EDAC (290 μ l stock) is added to the carboxymicroparticle reaction mixture, followed by 5.0 equivalents of sulfo N-hydroxysuccinimide (330 μ l stock). This mixture is allowed to incubate at room temperature for 2 hours and then a 2.0 molar equivalent of intercalator compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 (4 mg) is added at a concentration of 8 mg/400 μ l, or 2.0 mg/100 μ l in pH 8.0 0.1 N NaCl 0.1N Pi phosphate buffer. N-hydroxysuccinimide (Pierce) can be substituted for sulfo N-hydroxysuccinimide if it is first dissolved in a stock of DMF (Dimethyl formamide) and aliquoted as described above. After allowing 24 hours for complete reaction, the free dye is then removed by centrifugation, removal of mother liquor, and resuspension for several attempts until the solution went clear and no more dye is extracted from the samples. The purified capture reagent is then diluted to a stock of 2-4% solids in H₂O.

15

EXAMPLE 31

Solid Phase DNA Capture using Solid Phase Derived From Compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90

20

The capture of DNA onto solid phase can be performed using derivatization methods as described in EXAMPLE 5 except substituting the appropriate solid phase synthesized from the respective intercalator 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 as described in EXAMPLE 30.

25

Other appropriate solid phases such as carboxylated polystyrene microparticles or carboxylated magnetic microparticles can are also used instead of CM Sepharose.

30

EXAMPLE 32

Protocol for DNA Capture by Intercalator Modified Solid Phase Modified By Compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90

5 DNA can be released from solid phase as described in EXAMPLE 6 except that corresponding solid phase derived from intercalator compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 is used rather than the PTA 24 derivatized intercalator.

10

EXAMPLE 33

Fluorescence Staining in a Flow Cytometric Study of Chicken Erythrocyte Nuclei (CEN)

Protocol: 50 μ l of whole blood sample from two in-house donors and
15 3 μ l of CEN suspension is added to 1.0 ml of pre-warmed at 40°C WBC DIL without and with the compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80 at 1 μ g/ml concentration, mixed, introduced to the FACScan™ and 20" readings is acquired. Chicken erythrocyte nuclei (CEN) is used to measure the
20 brightness of the FL3 staining (mean FL3 of CEN). The whole blood samples used is about 4-5 hours old.

EXAMPLE 34

25 Viability Dyes on the Coulter Elite Flow Cytometer Using Compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80

Cell Isolation Protocol: Each tube of ficol isolated cells can be treated as follows: PBS with 0.1% NaAzide and 1.0% albumin (Sigma catalogue
30 #1000-3) Ficol specific gravity 1.119 (Sigma Histopague catalogue #1119-1).

- 10 ml of whole blood (EDTA anticoagulant) is diluted with 10 ml of PBSW. Into 4, 15 ml conical bottom tubes, 5 ml of the diluted blood is layered over 5 ml of ficol. The tubes is spun for 30 minutes at 400 x G. The interface layer which contains the lymphocytes, monocytes, granulocytes and platelets is aspirated and washed once in 5 ml PBS, by centrifuging tubes at 300 x G for 6 minutes. The cell pellet is resuspended in PBS, cells counted, and adjusted to 8.5×10^6 cells per ml.

Cell Staining Protocol:

- 10 Dye or compound solutions:

Compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80 - Stock solution 10 $\mu\text{g/ml}$ made by dissolving dye in PBS with 0.1 % NaAzide.

- 15 Staining of compound 1, 2, 3, 25, 26, 27, 28b, 29b, 30b, 31b, 32b, 33b, 34b, 35b, 36b, 37b, 38, 39b, 40b, 41b, 42b, or 80:

In 12 x 75 mm tube, 23.5 μl of cells is gently mixed with 76 μl of the compound stock solution. After 20 seconds, the tube is placed on Elite flow cytometer and data collected.

- 20 Flow cytometer protocol: Cells analyzed on the Elite flow cytometer (Coulter Electronics, Inc.).

- 25 Samples can be excited with an argon laser at 488 nm and 15 mW of power. Data is gated on the basis of size and granularity to exclude red blood cells, platelets and debris. The linear dye fluorescence of the gated distribution is analyzed using unstained cells as a control. The percent positive events (dead cells) and the mean fluorescence of the dead cell distribution is recorded.

EXAMPLE 35Conjugates of Intercalator Derivatized Antibodies and
Intercalator Derivatized Alkaline Phosphatase Using Double
Stranded Nucleic Acids as Conjugation Templates

5 Complementary strands of oligonucleotides, sense and antisense 14 to 20-mers, can be synthesized on a fully automated DNA synthesizer and purified as described in Bioconjugate Chemistry, 4, pp. 94-102, (1993). The synthesis of the conjugate between the enzyme calf intestinal alkaline phosphatase and IgG can be accomplished as follows.

10 a) Derivatization of the Intercalator PTA 24 with 30 Atom Heterobifunctional Linker Arm, 4-[(N-maleimidomethyl)tricaproamido]cyclohexane-1-carboxylate (SMTCC).

PTA 24, 0.100g (2.3×10^{-4} mole), is dissolved in 5 ml of 50% aqueous DMF (dimethylformamide). 30 atom heterobifunctional linker, 4-
15 [(N-maleimidomethyl)tricaproamido]cyclohexane-1-carboxylate (SMTCC), 0.157 g, (2.3×10^{-4} mole) synthesized as described in Bieniarz et al., US Patent Nos. 4,994,385, 5,002,883, 5,053,520, and 5,063,109 is dissolved in 3 ml of DMF and is added in one portion to the solution and stirred over 24 h at ambient temperature. The resulting maleimide derivatized PTA 24
20 contains is purified on silica gel column using 10% methanolic acetone as eluent.

b) Derivatization of the Calf Intestinal Alkaline Phosphatase with Iminothiolane.

Calf intestinal alkaline phosphatase, 6 mg (4×10^{-9} mole) in 1 ml of
25 PBS buffer is thiolated by treatment with a 450-fold molar excess of iminothiolane (164 ml of a 15 mg/ml solution in PBS buffer) for 30 min at ambient temperature. Excess reagent is removed by gel filtration with a Sephadex G-25 column (1x45 cm) equilibrated with PBS buffer. The fractions containing the derivatized enzyme are pooled and the concentration
30 of the enzyme in the pool is calculated by absorbance at 280 nm.

c) Conjugation of the SMTCC Derivatized PTA 24 to the Thiolated Calf Intestinal Alkaline Phosphatase from b).

The thiolated calf intestinal alkaline phosphatase from part b) and maleimide derivatized PTA 24 from part a) are combined at molar ratio 1:1 and incubated 18 h at 5°C. Unreacted thiol groups are capped by addition of 5 mM *N*-ethylmaleimide to 0.3mM final concentration followed by 1 h incubation at ambient temperature.

d) Derivatization of IgG with thiolates using site-specific functionalization of the Fc region of IgG.

Site-specific introduction of thiolates into Fc region of IgG is accomplished as described in Bieniarz et al., US Patent No. 5,191,066. In that way, between 4 and 10 thiolates are introduced into Fc region of the IgG.

e) Derivatization of the Fc Thiolated IgG from part d) with maleimide derivatized PTA 24 from part a).

The Fc thiolated IgG from part d) and maleimide derivatized PTA 24 from part a) are combined at molar ratio of 1:5 of thiolated IgG to SMTCC derivatized PTA 24. The solution is incubated in phosphate buffer pH 7.0 for 18 h at 5°C and the conjugate is purified on gel filtration column Sephadex G-25. Fractions containing proteins are pooled and concentrated using Amicon concentrator. Protein content of the final solution is established using Coomassie Dye Binding Assay, from Pierce Company.

f) Conjugation of the PTA 24 derivatized IgG from part e) to the PTA 24 derivatized calf intestinal alkaline phosphatase from part c) using double stranded 20-mer oligonucleotide composed of the complementary strands of the oligos.

Complementary strands of oligonucleotides are hybridized and examined as described in Bioconjugate Chemistry, 4, pp. 94-102, (1993). PTA 24 derivatized IgG from part e), PTA 24 derivatized calf intestinal alkaline phosphatase and double stranded oligonucleotide 20-mer used as the conjugation template are incubated overnight at pH 7.0 for 18 h in molar

ratios of 1:1:1 at ambient temperature. The conjugate is filtered through a gel filtration column G-25. The fractions are assayed for alkaline phosphatase activity using fluorogenic substrate 4-methylumbelliferyl phosphate, and for the antibody activity using the antiidiotypic IgG labelled with fluorescein.

The conjugate is also examined by gel filtration HPLC columns.

EXAMPLE 36

Conjugation of Intercalator Derivatized Liposome to a Double Stranded DNA

Preparation of the liposomes is carried as described in Fiechtner et al., US Patent No. 4,912,208. These liposomes display on their surface primary amines because they are prepared from diphosphatidylethanolamine lipids. The preparation of the liposomes is carried in presence of membrane impermeable fluorescent dyes disclosed in US Patent No. 4,912,208. Thus, the molecules of the fluorescent dyes are substantially at very high, self quenching concentrations inside the liposomes and consequently they are nonfluorescent. The surface amines of the liposomes are derivatized with thiolates by essentially the same method used to introduce thiolates into alkaline phosphatase described in the EXAMPLE 35. PTA 24 and other intercalators disclosed in the preceding examples are derivatized with maleimides, utilizing SMTCC reagent, as described in EXAMPLE 35. Thiolate derivatized liposomes and maleimide derivatized PTA 24 or other intercalators are incubated together in a vessel under conditions essentially identical to the ones described in EXAMPLE 35. The fluorescent dye containing liposomes derivatized with multiple intercalators are incubated at pH ranging from 5 to 9, preferably 7.0 with the sample containing small concentrations of the double stranded DNA coated or immobilized on a solid phase. The incubation is followed by wash and addition of detergent. The

concomitant lysis of the liposome membrane results in spilling of the fluorogenic dye, dequenching of the fluorescence and appearance of signal.

Alternatively, the probe multimeric single stranded DNA may be immobilized on a solid phase; the patient sample suspected to contain a complementary single stranded DNA is incubated in presence of the probe; the liposome-intercalator is brought in contact with the hybridized sample and the contents of the vessel are incubated and washed several times to remove excess of the PTA 24 derivatized liposomes. If the probe finds a complementary sequence in the patient sample, the addition of the detergent results in lysis of the liposomes and signal. If however the probe and patient sample DNA strands are not complementary, no double stranded DNA is present and substantially all the PTA 24 derivatized liposome is washed away from the solid phase, resulting in no signal.

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EXAMPLE 37

Detection of DNA Using A Fluorescent Intercalator Conjugate Derived From Intercalator Compound 24, 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90

The synthesis of intercalator maleimide functionalized intercalator can be effected by the procedure as already described in EXAMPLE 35 except that the intercalator compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 is used in place of PTA 24. An appropriate signal generating entity such as phycoerythrin or allophycocyanine is covalently attached to this maleimide derivatized intercalator through a thiolate on the thiolated protein prepared as described in EXAMPLE 35. After allowing time for binding of the intercalator portion of the conjugate to the immobilized ds-DNA molecule and washing away the unbound conjugate, then double stranded DNA is detected on the solid phase or other immobilized entity using the fluorescence of the phycoerythrin that is localized by the binding of the intercalator portion to

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the double stranded DNA molecule. The fluorescence emission is read at approximately 580 nm using methods known to those skilled in the art while exciting the fluorescent protein at a wavelength that is appropriate for efficient excitation as is determined by one skilled in the art.

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EXAMPLE 38

Detection of DNA Using an Enzyme Intercalator Conjugate Derived From Intercalator Compound 24, 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90

10

The synthesis of intercalator maleimide functionalized intercalator can be effected by the procedure as already described in EXAMPLE 35 except that the intercalator compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 is used in place of PTA 24. An appropriate signal generating entity such as alkaline phosphatase, β -galactosidase, esterase, or β -lactamase is covalently attached to this maleimide derivatized intercalator through the thiolate of the thiolated protein that is prepared as described in EXAMPLE 35. After allowing time for binding of the intercalator portion of the conjugate to the immobilized ds-DNA molecule and washing away the unbound conjugate, then double stranded DNA is detected on the solid phase or other immobilized entity such as a colloid or microparticle by fluorescence or chemiluminescence afforded by the turn-over of a non-fluorescent or non-chemiluminescent substrate of the appropriate enzyme to a fluorescent or chemiluminescent entity respectively as is determined by one skilled in the art. The fluorescence emission or chemiluminescence is then read at the appropriate wavelengths by using standard methods of fluorescence excitation or detection of chemiluminescence respectively that are known to those skilled in the art.

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EXAMPLE 39

Detection of DNA Using an Intercalator - Chemiluminophore Conjugate Derived from Intercalator Compound 24, 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90

5

The synthesis of intercalator maleimide functionalized intercalator can be effected by the procedure as already described in EXAMPLE 35 except that the intercalator compound 25, 26, 27, 29b, 35b, 38b, 50, 54a, 54c, 58a, 58c, 64, 68a, 68c, 71a, 71c, 80, 85, or 90 is used in place of PTA 24. An appropriate activatable chemiluminescent signal generating entity such as acridinium sulfonamide is covalently attached to this intercalator by thiolate of the chemiluminescent entity that is prepared so as to be reactive towards the maleimide such as can be devised by one skilled in the art. After binding of ds-DNA with the intercalator -

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chemiluminophore conjugate, the excess or unbound conjugate is washed away and double stranded DNA is then detected on the solid phase or other immobilized entity by direct chemiluminescence triggered by the addition of an appropriate activation reagent such as hydrogen peroxide leading to a chemiluminescent entity and chemiluminescence is then read at at the appropriate wavelengths by using standard methods of detection of chemiluminescence such as are known to those skilled in the art.

EXAMPLE 40

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Synthesis of Intercalator Derivatized Dendrimer

Intercalator derivatized dendrimer can be synthesized from Dendritic Polymers obtained from PolySciences. The intercalator bromide intermediates 22, 49, 58, 63, 71, 83, or 88 is used to alkylate the amines on the 3rd, 4th, 5th, 6th, 7th, 8th, 9th, or 10th generation dendrimer by

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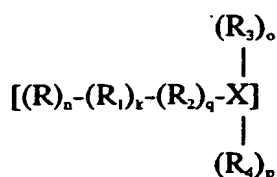
heating in dimethyl formamide or other appropriate organic solvent to between 30-90°C for 0.25 - 72 hours. The product is then purified in the

case of when intercalator compound 58, 63, or 71 is used by using size exclusion chromatography on a G-25 SephadexTM column in water or an appropriate aqueous buffer and the intercalator derivatized dendrimer is obtained. In the case of intercalator compound 22, 83, or 88, the column in
5 G-25 is run in water and the protected intercalator derivatized dendrimer is then isolated. This material is then subjected to heating to 90°C for 2-4 hours in 4N HCl to hydrolyze the aromatic amine protecting groups and then the solution is neutralized to pH 4-10 and a G-25 column is run in an appropriate buffer on the final product to obtain pure intercalator derivatized
10 dendrimer.

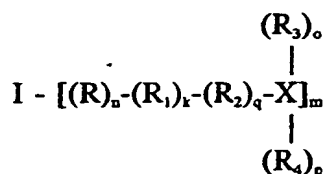
Although the present invention and its advantages have been described in detail, those skilled in the art should understand that they can make various changes, substitutions and alterations herein without departing from the spirit and scope of the invention as defined by the appended
15 claims.

CLAIMS

1. A compound comprising an I moiety bonded to one or more T moiety, said T moieties, when more than one T moiety are present, are the same or different from one another, said T moiety having the formula of:



and said compound having a formula:



wherein: I is an aromatic or heteroaromatic segment;

X is a heteratom selected from the group consisting of nitrogen and sulfur;

R, R₁ and R₂ are the same or different from one another and are alkyl, alicyclic, heteroalicyclic, aromatic or heteroaromatic groups;

R₃ and R₄ are hydrogen when X is nitrogen; R₃ and R₄ are methyl, ethyl, or phenyl groups when X is sulfur;

k is zero or an integer from 1 to 10;

q is zero or an integer from 1 to 10;

n is an integer from 2 to 20;

m is an integer from 1 to 5;

o is zero or one; and

p is zero or one;

and the acid addition salts thereof;

35 provided that said I moiety is not phenantridium when: X is nitrogen; R₃ and R₄ are hydrogens; R, R₁ and R₂ are methylene groups; o is 1; p is 1; m is 1; and the acid addition salts thereof;

provided that said I moiety is not phenantridium when: X is nitrogen; R₃ and R₄ are hydrogens; R, R₁ and R₂ are methylene groups; o is
40 1; p is 1; m is 2; a first said T moiety directly bonded to said I moiety in which n is 2, k is 1 and q is zero; a second said T moiety, directly bonded to said first T moiety, in which n is 2, and k and q are zero; and acid addition salts thereof;

provided that said I moiety is not phenantridium when: X is
45 nitrogen; R₃ and R₄ are hydrogens; R, R₁ and R₂ are methylene groups; o is 1; p is 1; m is 2; a first said T moiety directly bonded to said I moiety in which n is 2, k is 1 and q is zero; a second said T moiety, directly bonded to said first T moiety, in which n is 2, k is 1 and q is 1 and acid addition salts thereof; and

50 provided that said I moiety is not phenantridium when: X is nitrogen; R₃ and R₄ are hydrogen; o is 1; p is 1; m is 3; n is 2; k is 1; q is zero; and acid addition salts thereof.

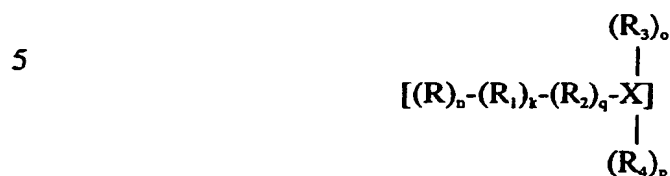
2. The compound of claim 1, wherein n is an interger from 2 to 3 and m is an interger from 1 to 3.

3. The compound according to claim 1, wherein R, R₁ and R₂ are the same or different from one another and are:
alkyl group of one to four carbons;
alicyclic group of five to six carbons;
heteroalicyclic group of three to five carbons and one or two
5 heteroatoms selected from the group consisting of nitrogen, oxygen or sulfur;

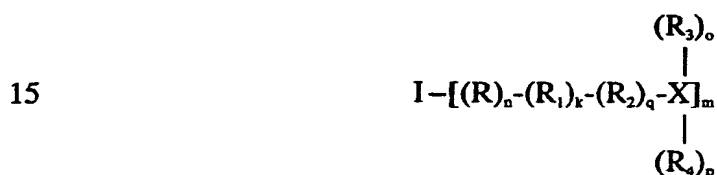
aromatic group selected from the group consisting of benzene, phenyl or naphthyl; or

heteroaromatic group of one to five carbons and one to four heteratoms selected from the group consisting of nitrogen, oxygen and sulfur.

4. A compound comprising an I moiety bonded to one or more T moiety, said T moieties, when more than one T moiety are present, are the same or different from one another, said T moiety having the formula of



and said compound having a formula:



wherein: I is an aromatic or heteroaromatic segment;

X is a heteratom selected from the group consisting of nitrogen and sulfur;

R, R₁ and R₂ are the same or different from one another and are alkyl group of one to four carbons; alicyclic group of five to six carbons; heteroalicyclic group of three to five carbons and one or two heteroatoms selected from the group consisting of nitrogen, oxygen or sulfur; aromatic group selected from the group consisting of benzene, phenyl or naphthyl; or heteroaromatic group of one to five carbons and one to four

heteroatoms selected from the group consisting of nitrogen, oxygen and
30 sulfur;

R_3 and R_4 are hydrogen when X is nitrogen; R_3 and R_4 are
methyl, ethyl, or phenyl groups when X is sulfur;

k is zero or an integer from 1 to 10;

q is zero or an integer from 1 to 10;

35 n is an integer from 2 to 20;

m is an integer from 1 to 5;

o is zero or one; and

p is zero or one;

and the acid addition salts thereof;

40 provided that said I moiety is not phenantridium when: X is
nitrogen; R_3 and R_4 are hydrogens; R , R_1 and R_2 are methylene groups; o is
1; p is 1; m is 1; and the acid addition salts thereof;

provided that said I moiety is not phenantridium when: X is
nitrogen; R_3 and R_4 are hydrogens; R , R_1 and R_2 are methylene groups; o is
45 1; p is 1; m is 2; a first said T moiety directly bonded to said I moiety in
which n is 2, k is 1 and q is zero; a second T moiety, directly bonded to
said first T moiety, in which n is 2, and k and q are zero; and acid addition
salts thereof;

provided that said I moiety is not phenantridium when: X is
50 nitrogen; R_3 and R_4 are hydrogens; R , R_1 and R_2 are methylene groups; o is
1; p is 1; m is 2; a first said T moiety directly bonded to said I moiety in
which n is 2, k is 1 and q is zero; a second T moiety, directly bonded to
said first T moiety, in which n is 2, k is 1 and q is 1 and acid addition salts
thereof; and

55 provided that said I moiety is not phenantridium when: X is
nitrogen; R_3 and R_4 are hydrogen; o is 1; p is 1; m is 3; n is 2; k is 1; q is
zero; and acid addition salts thereof.

5. The compound of claim 4, wherein n is an integer from 2 to 3 and m is an integer from 1 to 3.

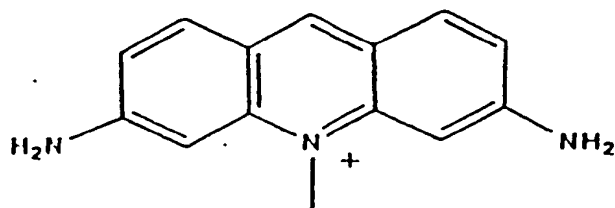
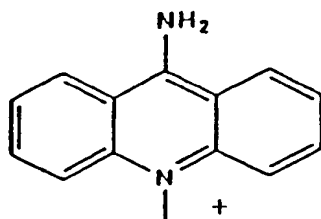
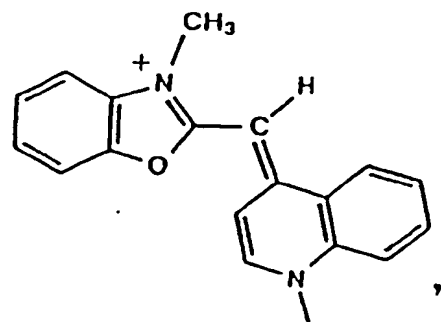
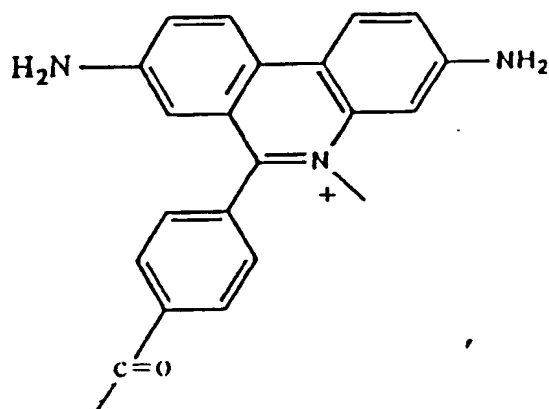
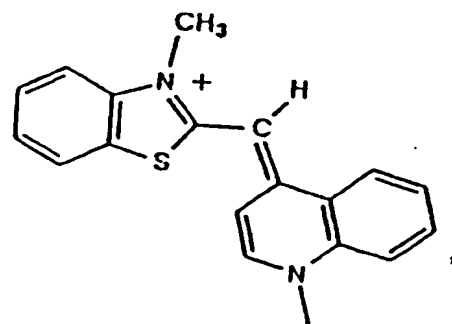
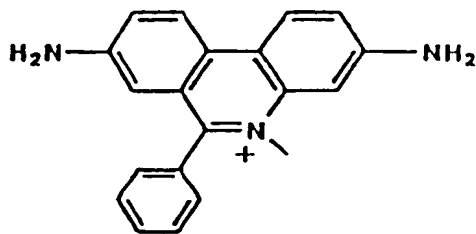
6. A compound comprising an I moiety bonded to one or more T moiety, said T moieties, when more than one said T moiety are present, are the same or different from one another, said compound having a formula

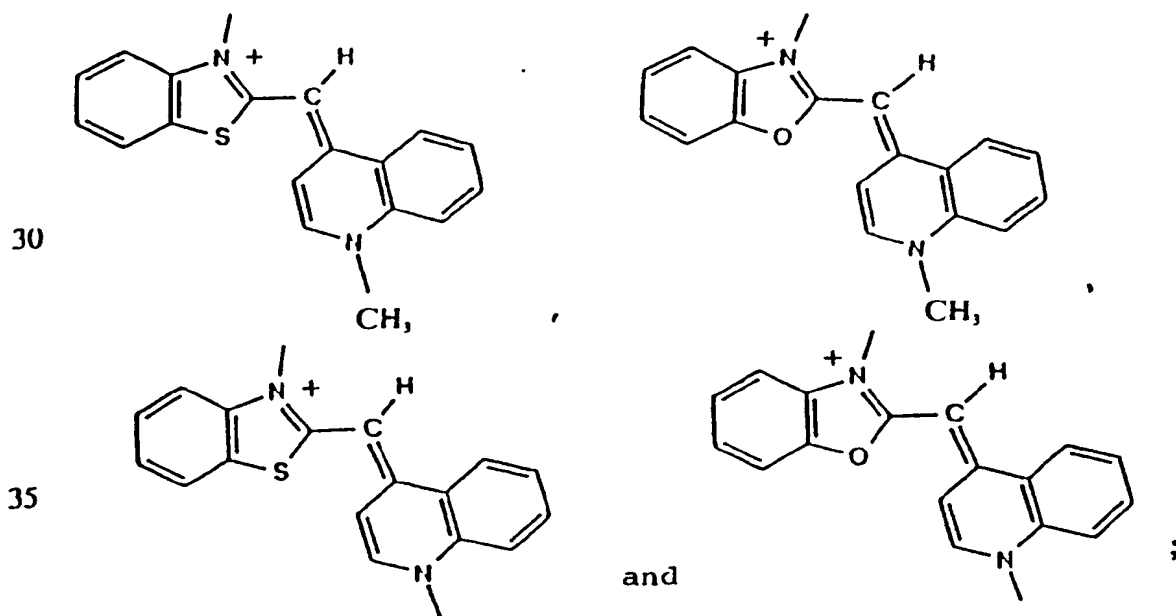


wherein m is an integer from 1 to 5;

wherein said I moiety is selected from the group consisting

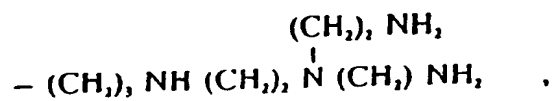
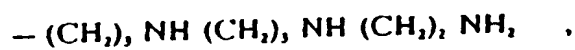
of:



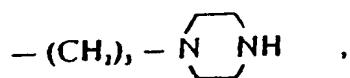
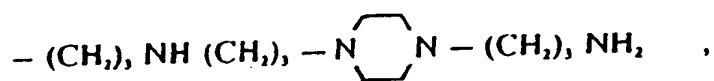


said T moiety is selected from the group consisting of:

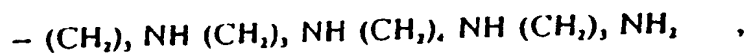
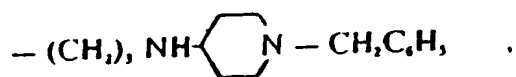
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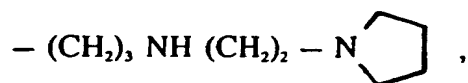
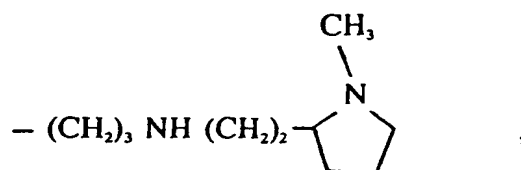
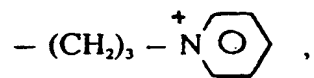


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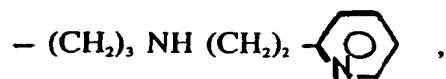
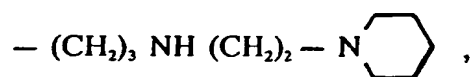


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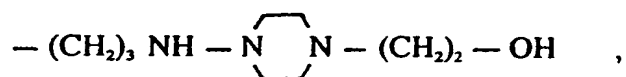
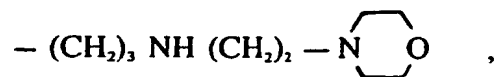
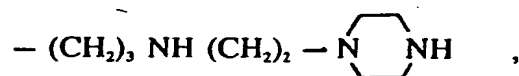
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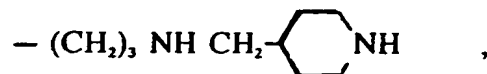
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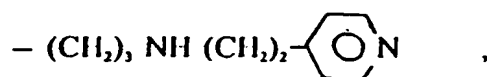
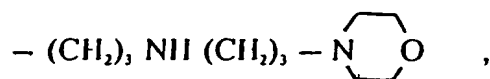
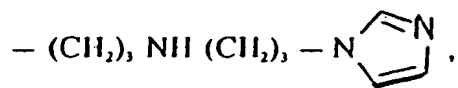


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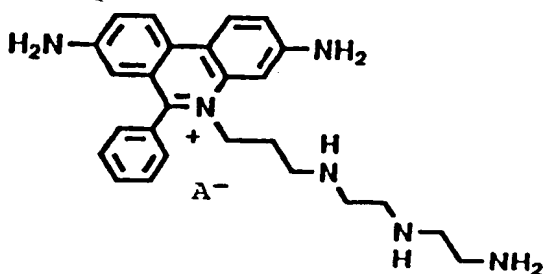
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and combination thereof.

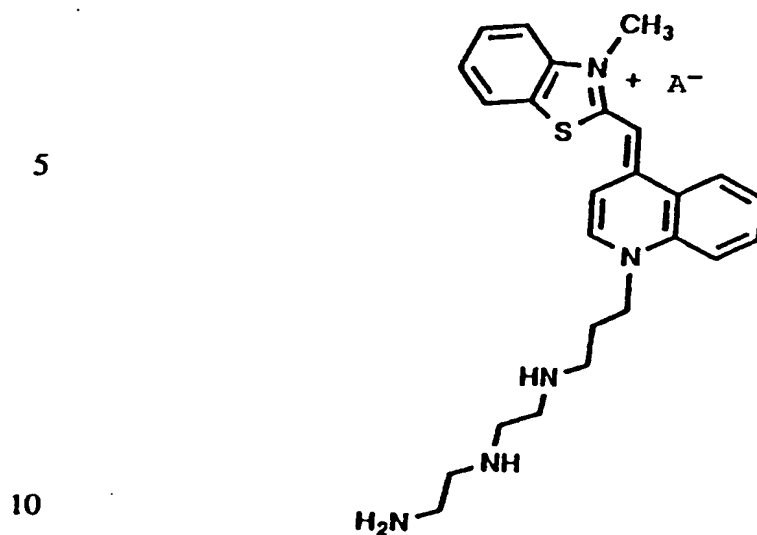
7. A compound having the formula



wherein A⁻ is an acceptable monvalent counter anion.

8. The compound of claim 7, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

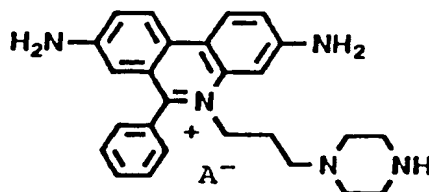
9. A compound having the formula



wherein A⁻ is an acceptable monvalent counter anion.

10. The compound of claim 9, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

11. A compound having the formula



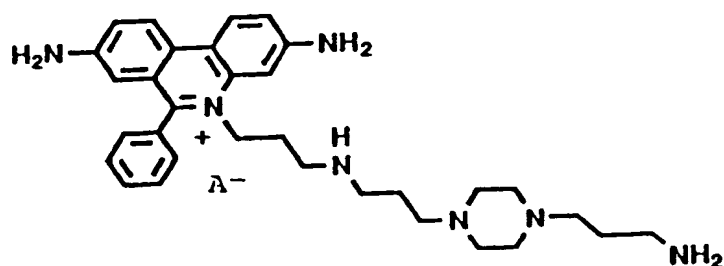
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wherein A⁻ is an acceptable monvalent counter anion.

12. The compound of claim 11, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

13. A compound having the formula

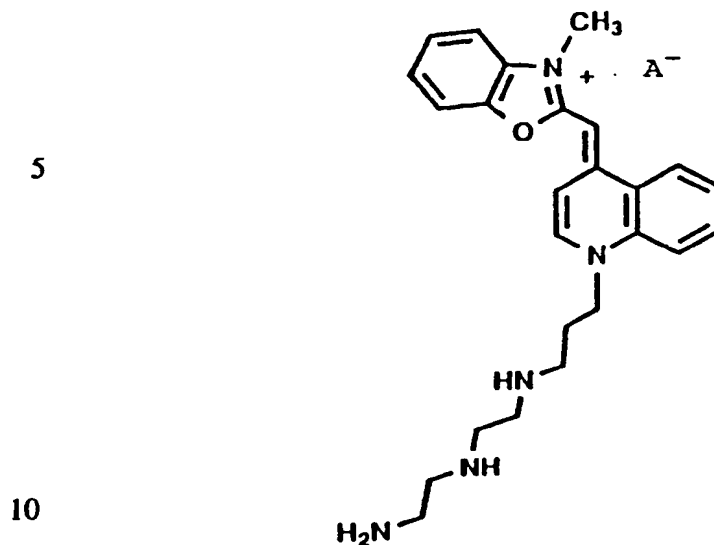
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wherein A^- is an acceptable monovalent counter anion.

14. The compound of claim 13, wherein said A^- is selected from the group consisting of chloride, bromide and iodide.

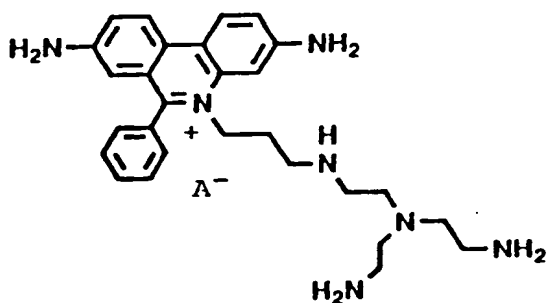
15. A compound having the formula



wherein A⁻ is an acceptable monvalent counter anion.

16. The compound of claim 15, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

17. A compound having the formula

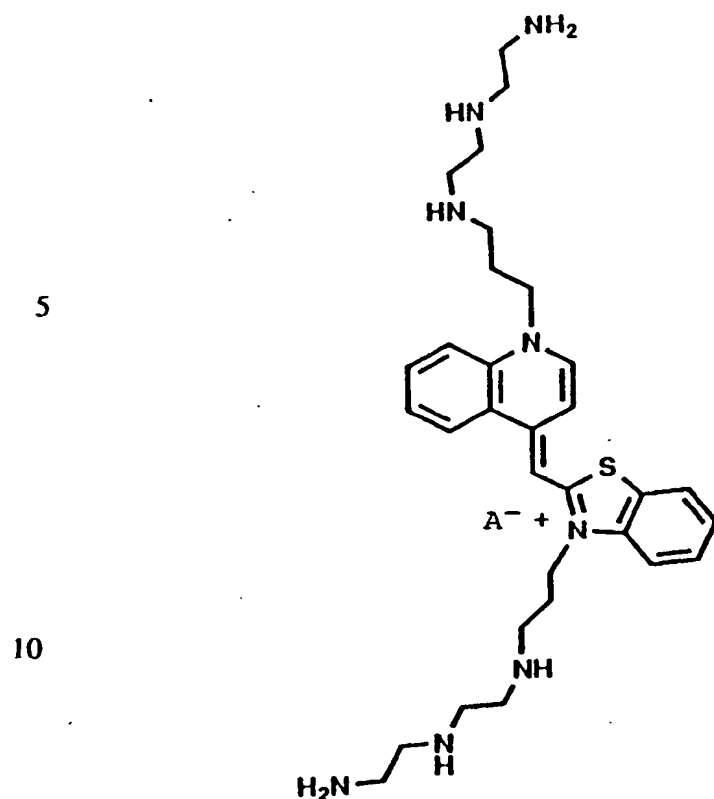


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wherein A⁻ is an acceptable monvalent counter anion.

18. The compound of claim 17, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

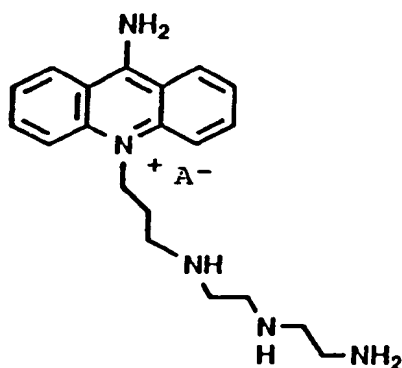
19. A compound having the formula



wherein A^- is an acceptable monvalent counter anion.

20. The compound of claim 19, wherein said A^- is selected from the group consisting of chloride, bromide and iodide.

21. A compound having the formula

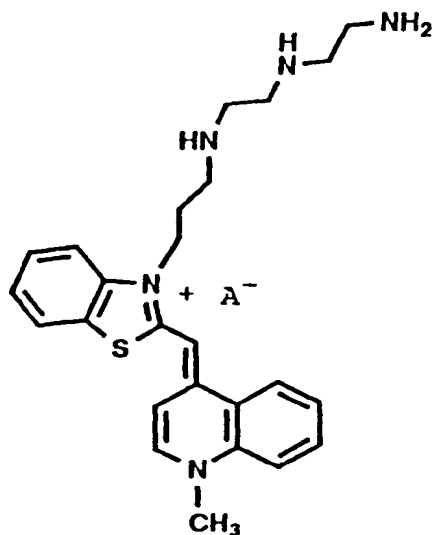


wherein A^- is an acceptable monvalent counter anion.

22. The compound of claim 21, wherein said A^- is selected from the group consisting of chloride, bromide and iodide.

23. A compound having the formula

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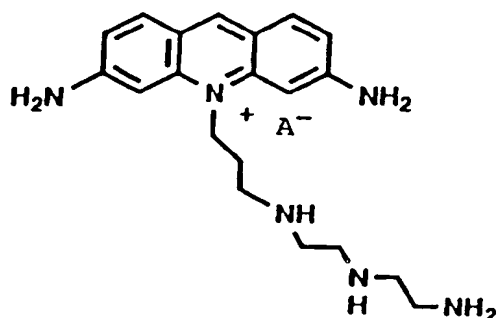


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wherein A^- is an acceptable monvalent counter anion.

24. The compound of claim 23, wherein said A^- is selected from the group consisting of chloride, bromide and iodide.

25. A compound having the formula



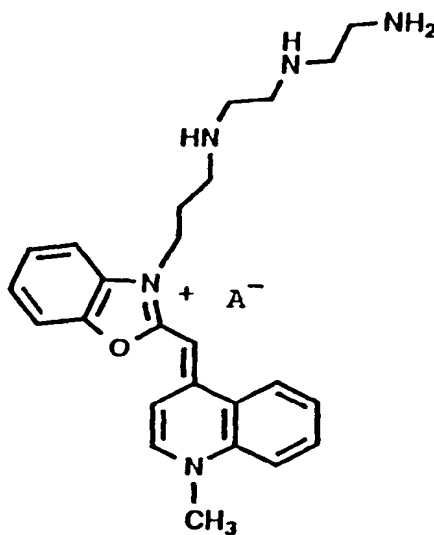
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wherein A⁻ is an acceptable monvalent counter anion.

26. The compound of claim 25, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

27. A compound having the formula

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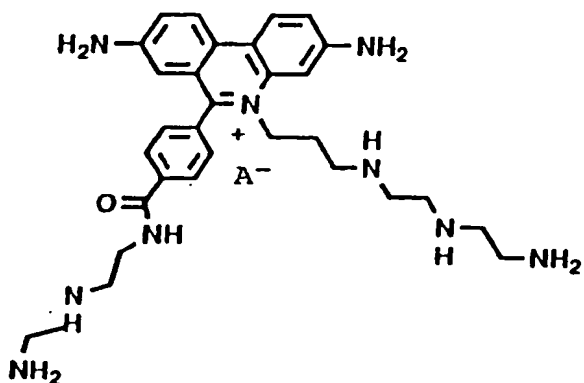
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wherein A⁻ is an acceptable monovalent counter anion.

28. The compound of claim 27, wherein said A⁻ is selected from the group consisting of chloride, bromide and iodide.

29. A compound having the formula

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wherein A^- is an acceptable monvalent counter anion.

30. The compound of claim 29, wherein said A^- is selected from the group consisting of chloride, bromide and iodide.

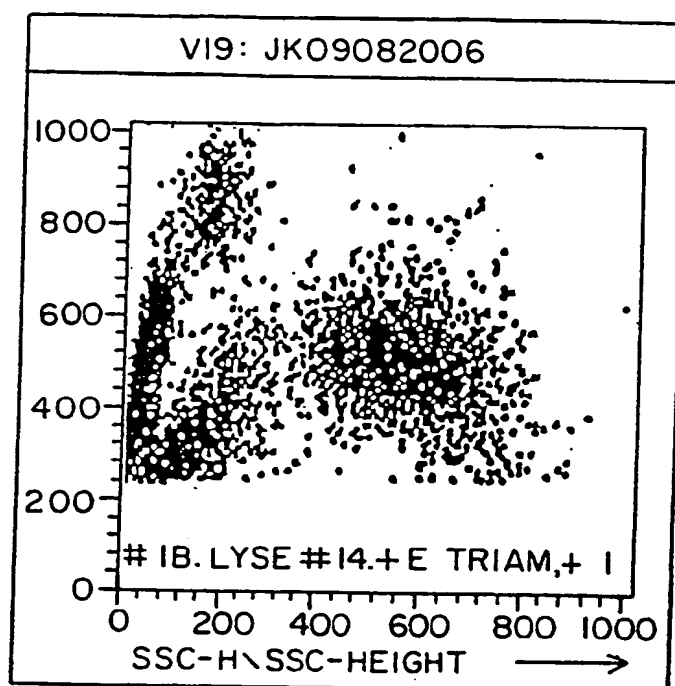


FIG. 1

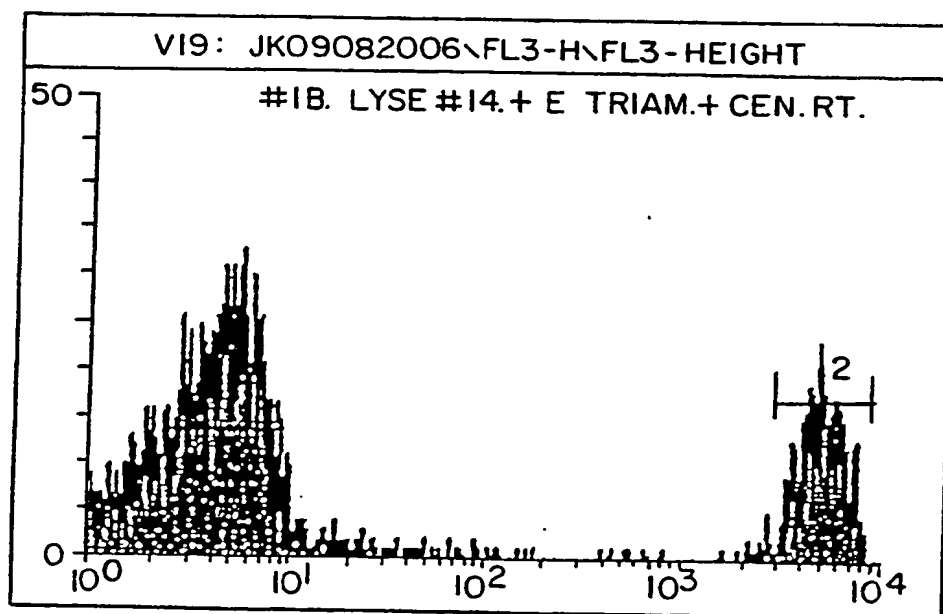


FIG. 2

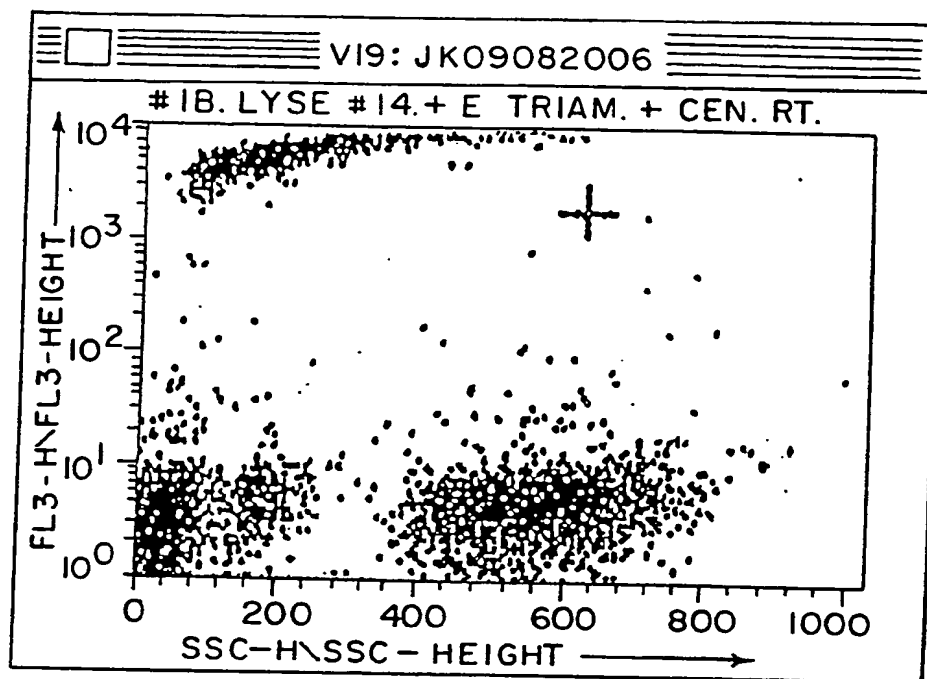


FIG. 3

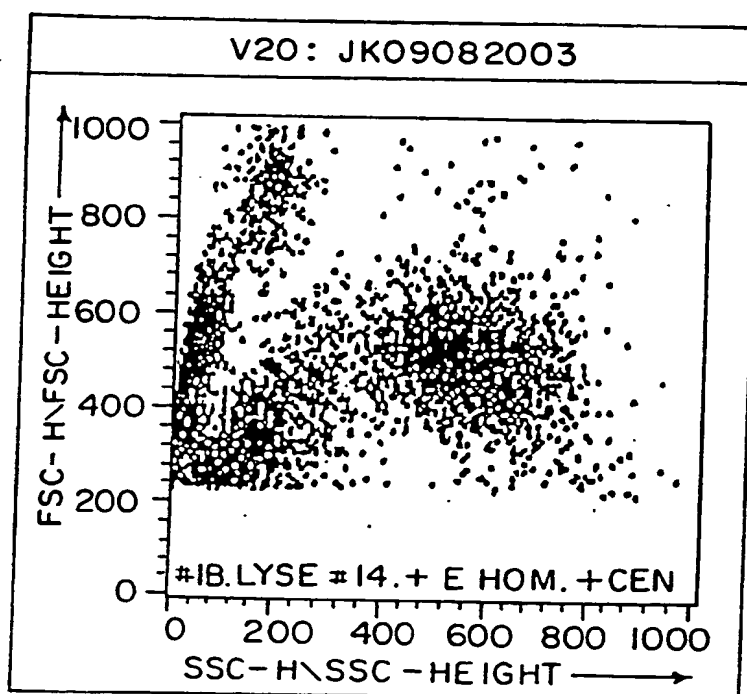


FIG. 4

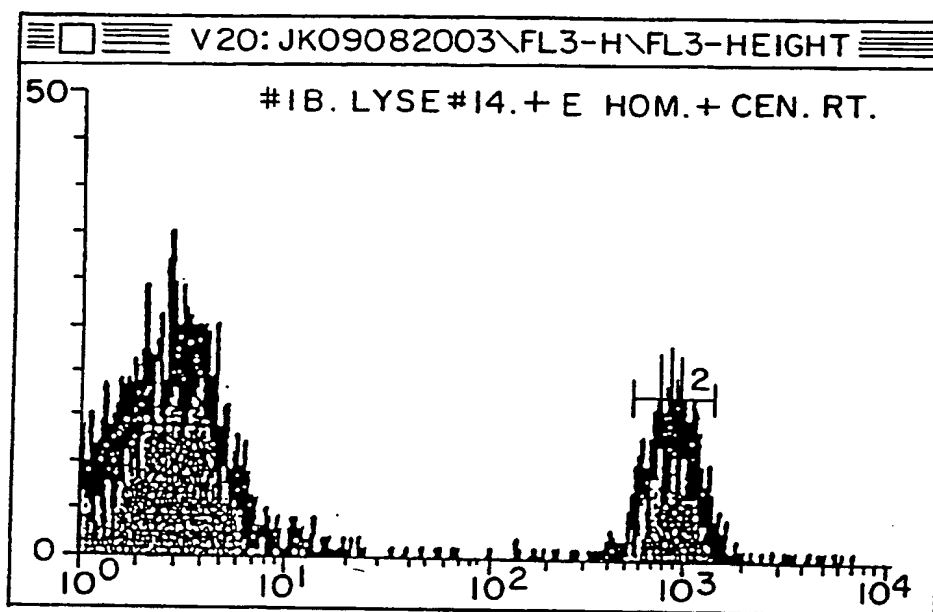


FIG.5

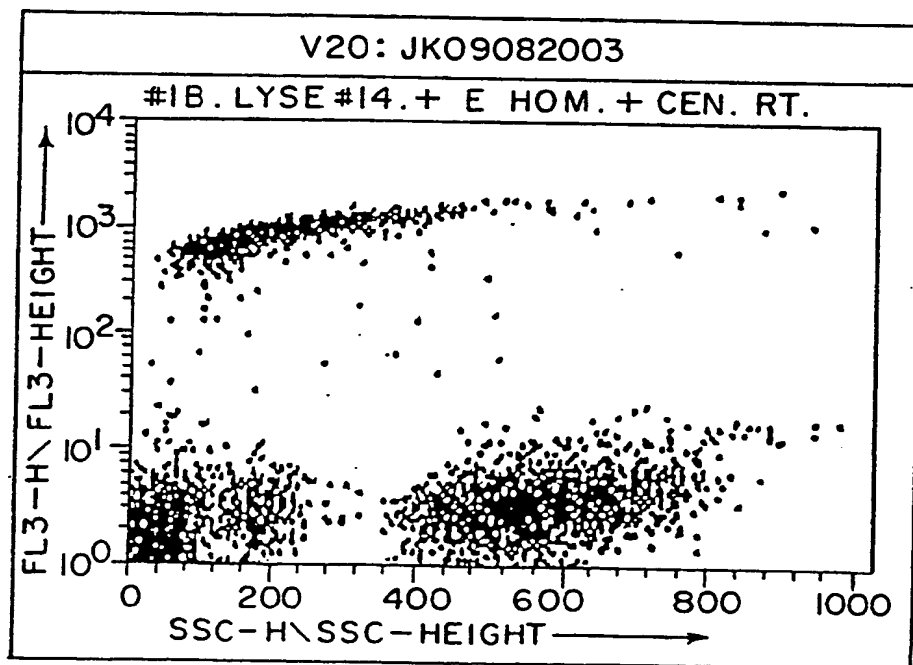


FIG.6

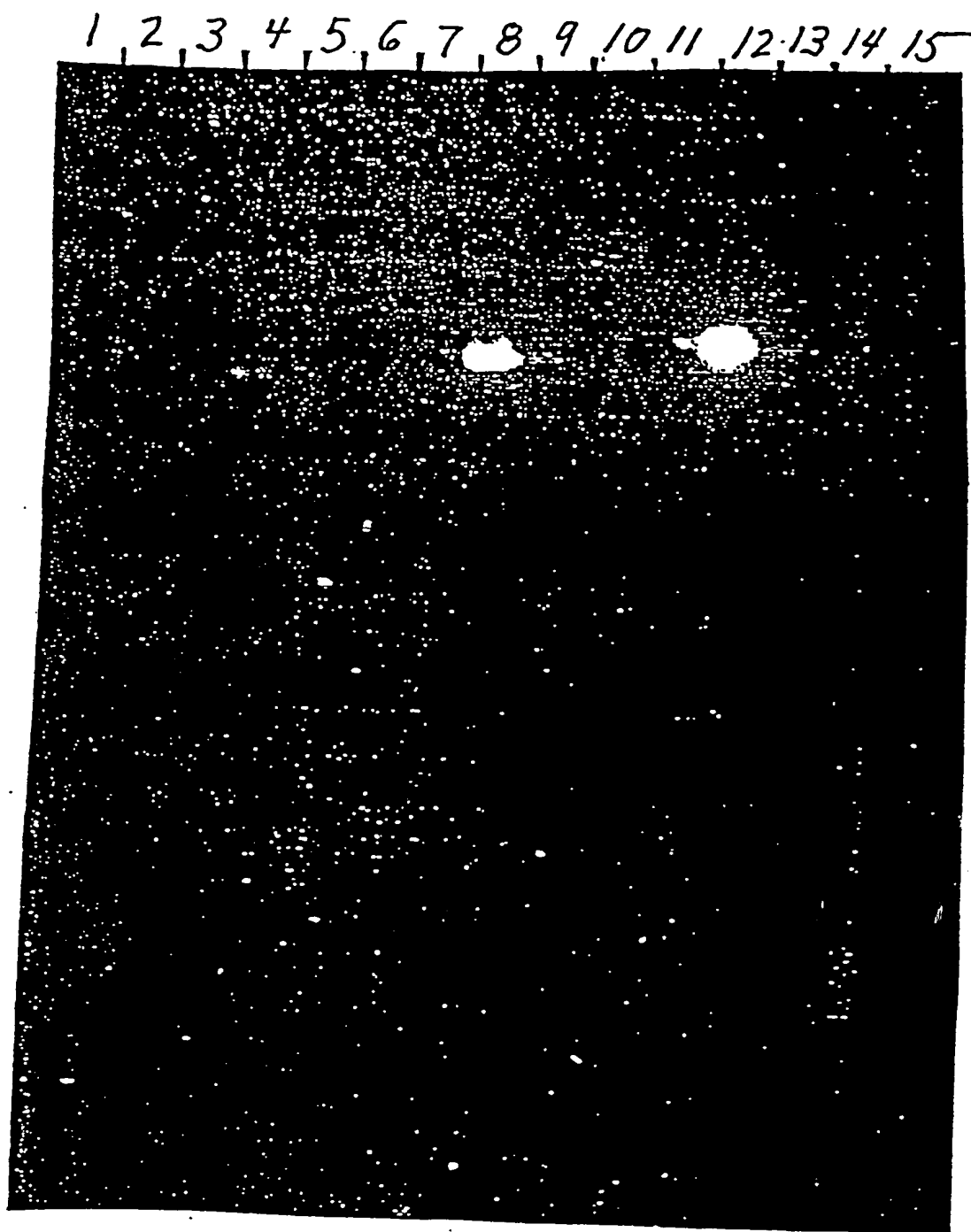


Fig. 7

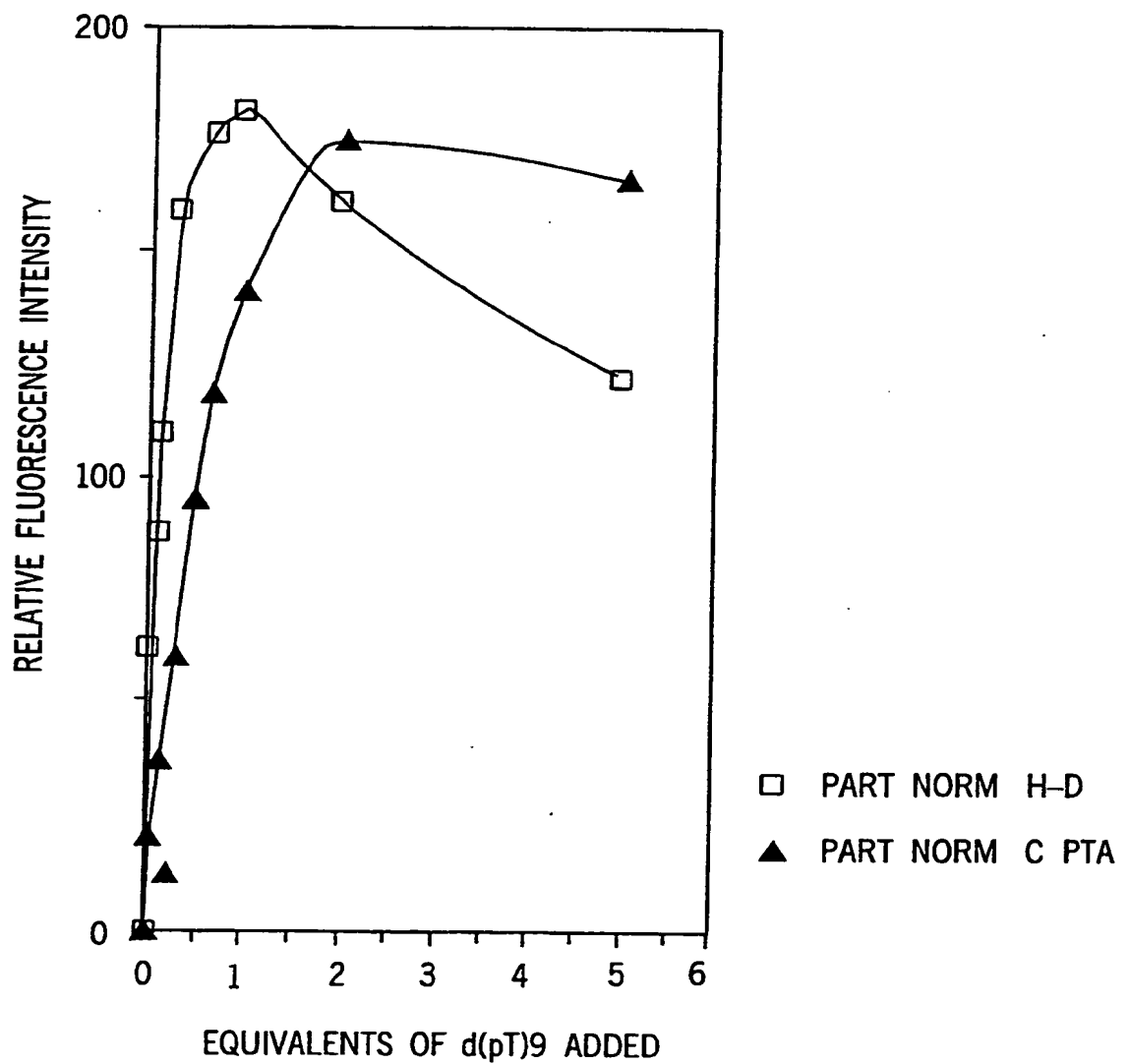


FIG. 8

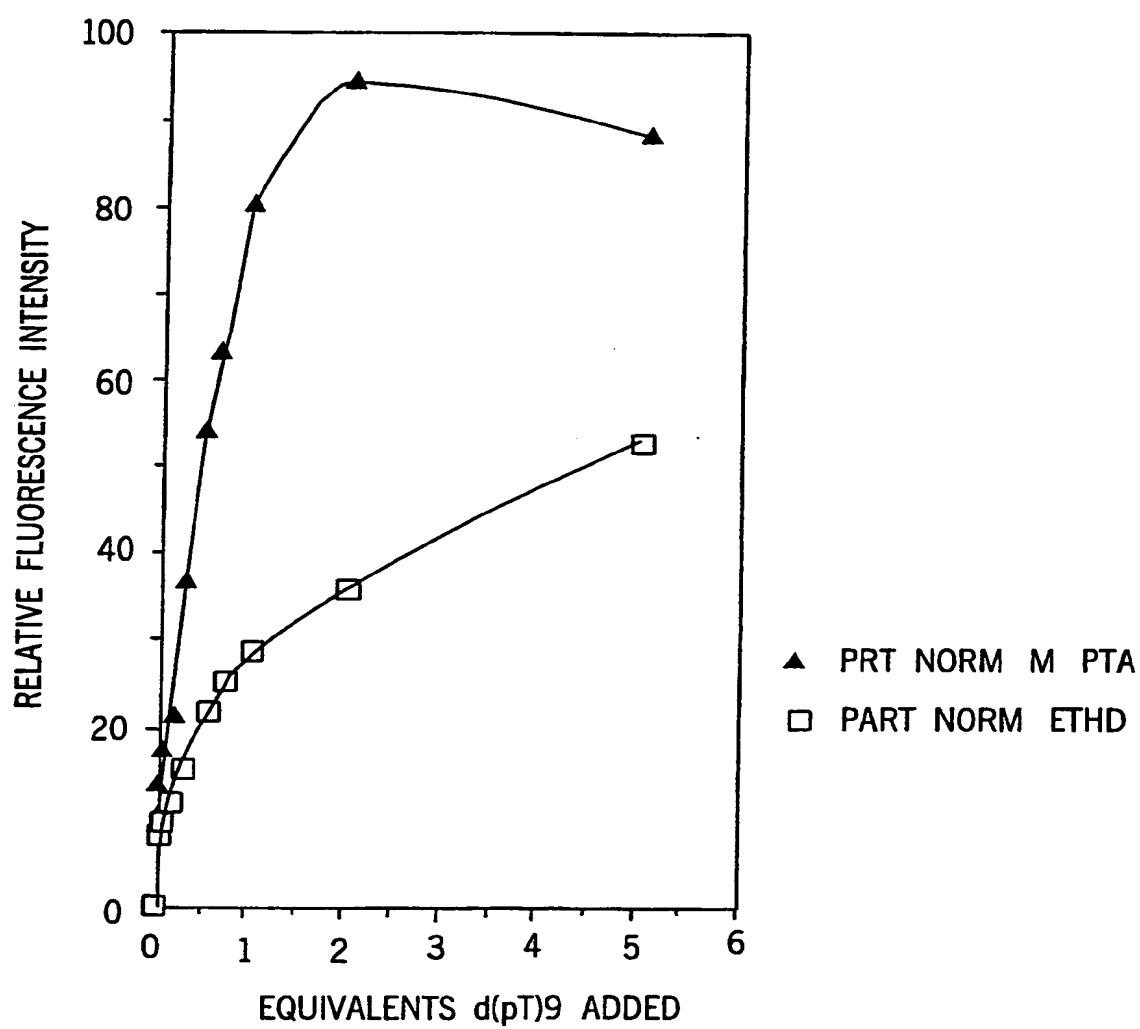


FIG. 9

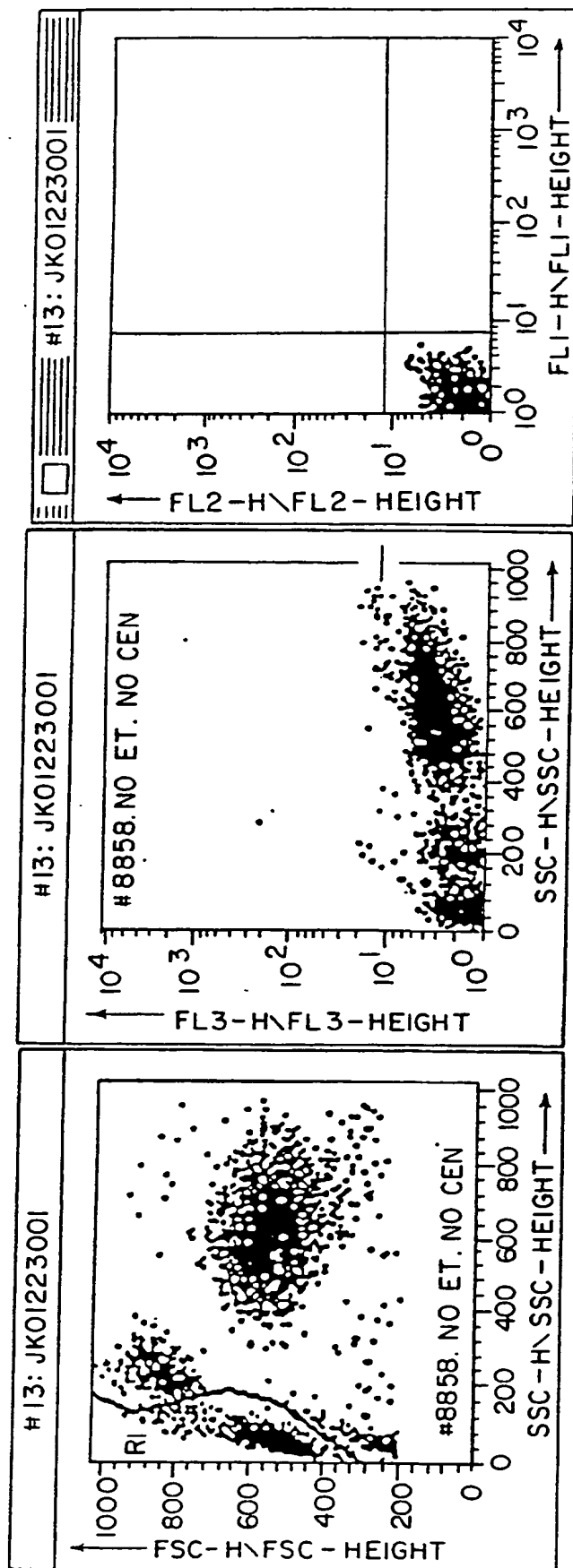


FIG.10A

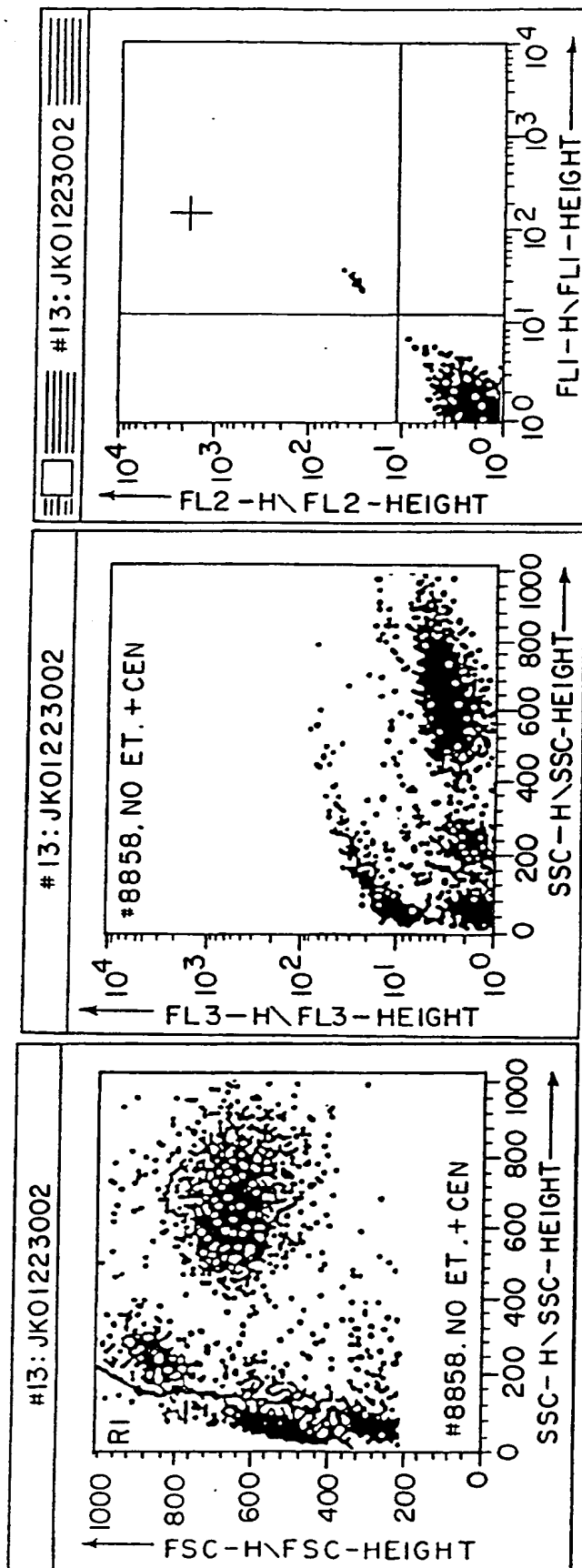


FIG.10B

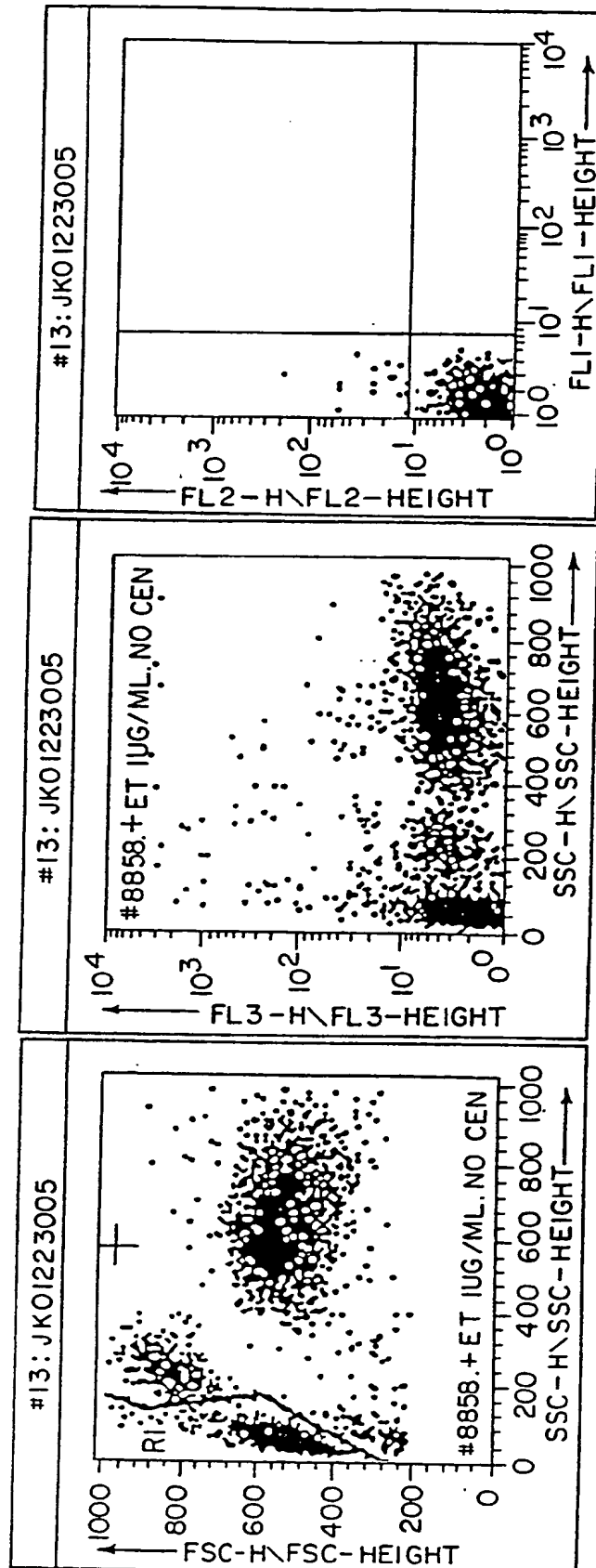


FIG.10C

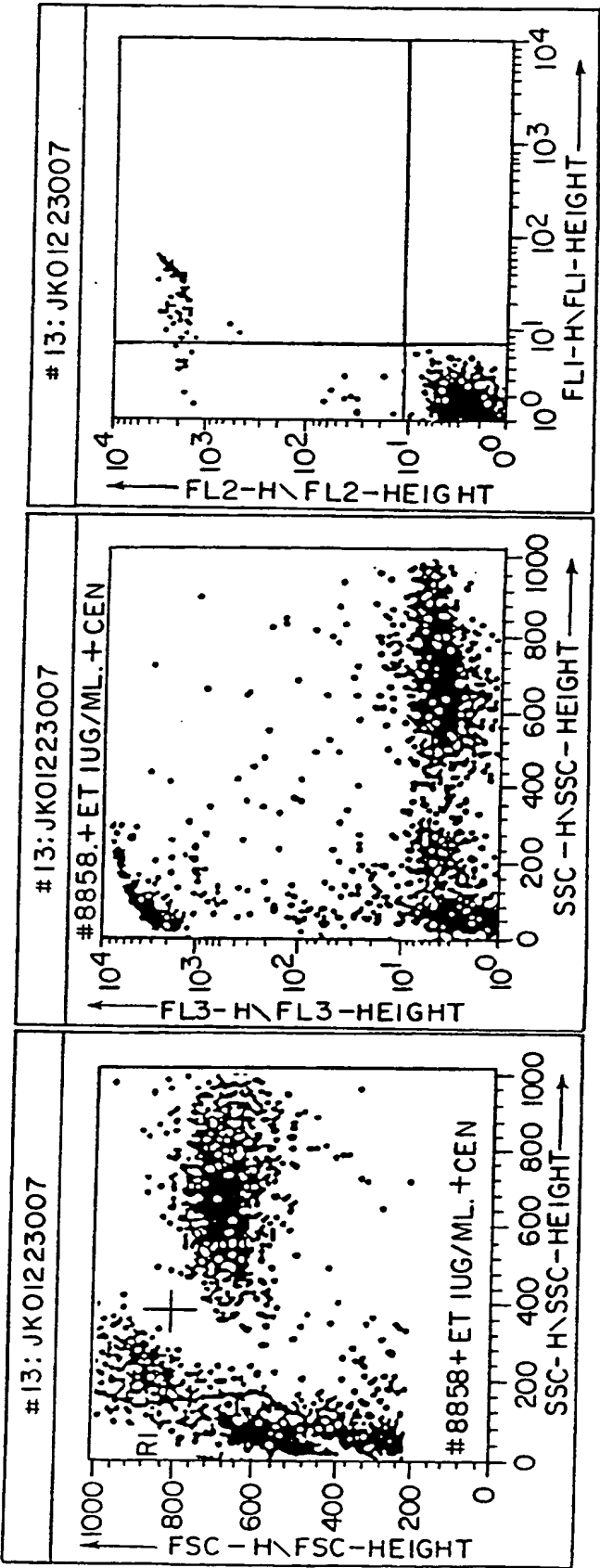


FIG.10D

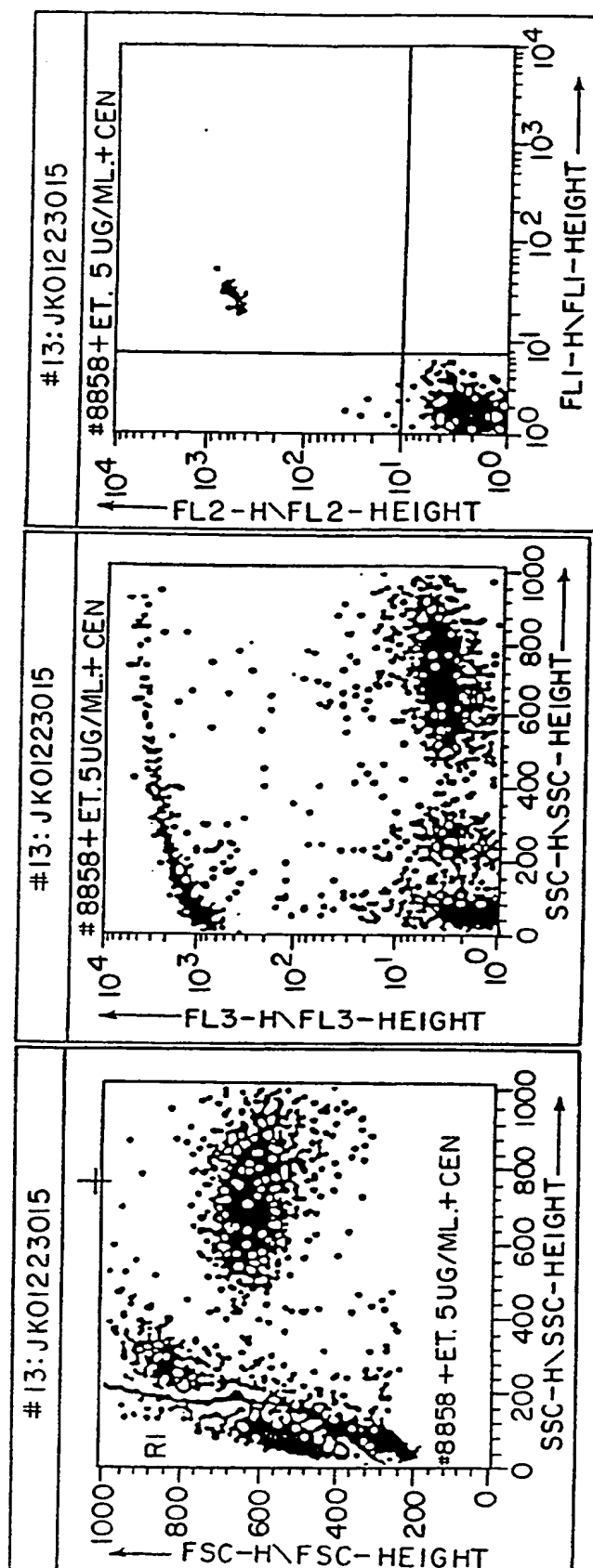


FIG.10E

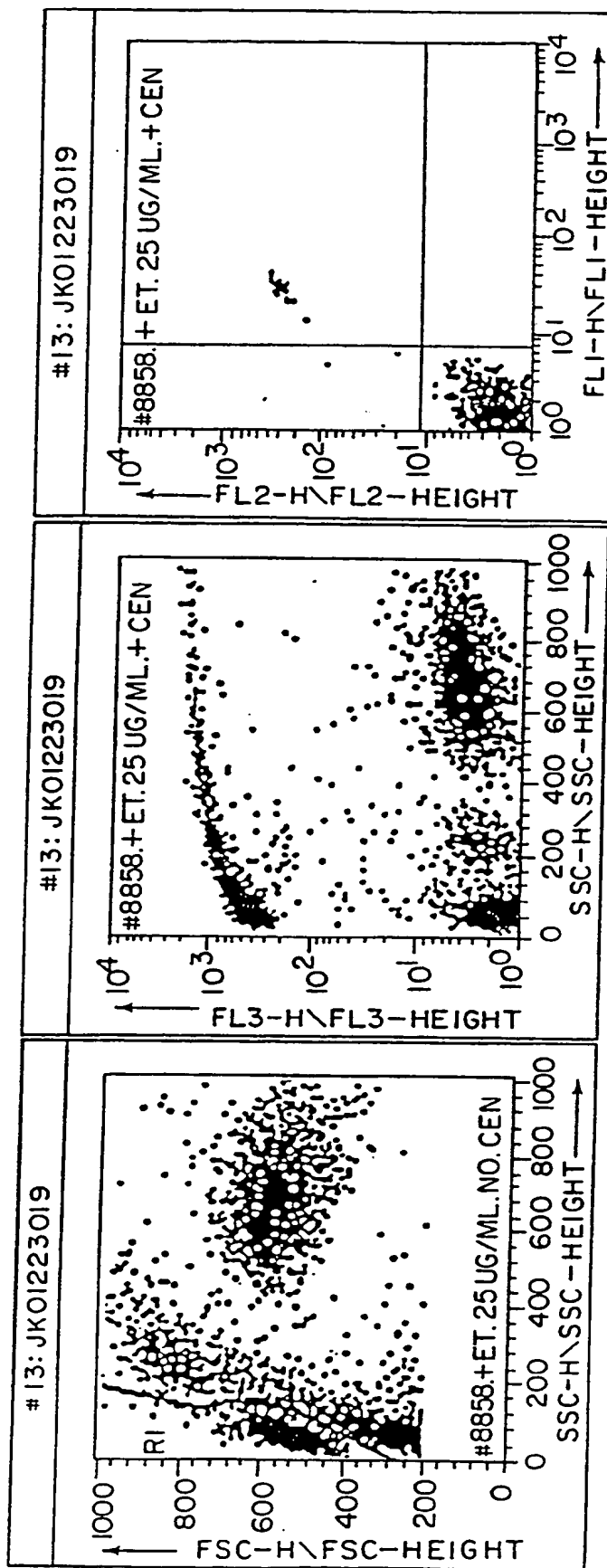


FIG.10F

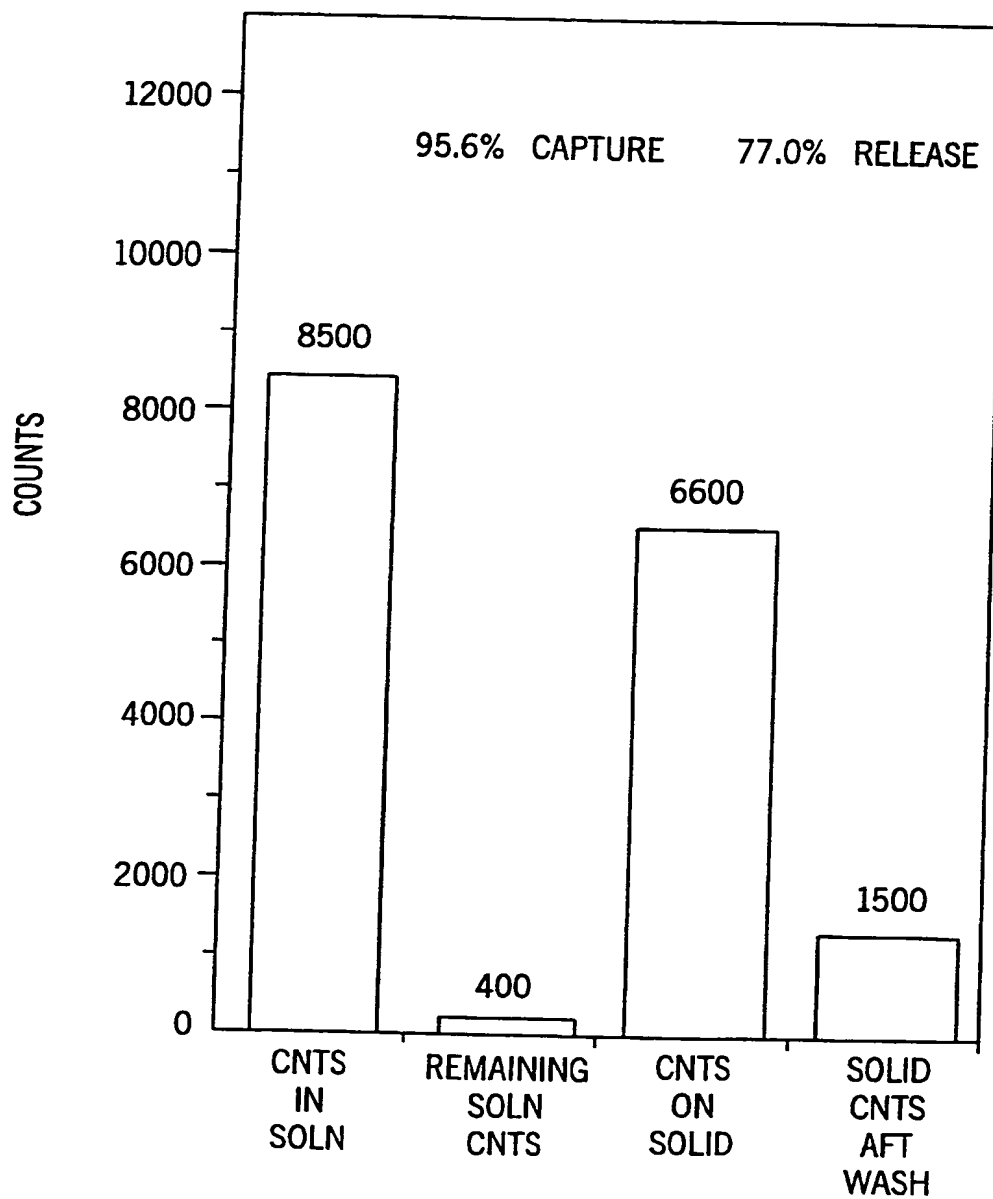


FIG. 11

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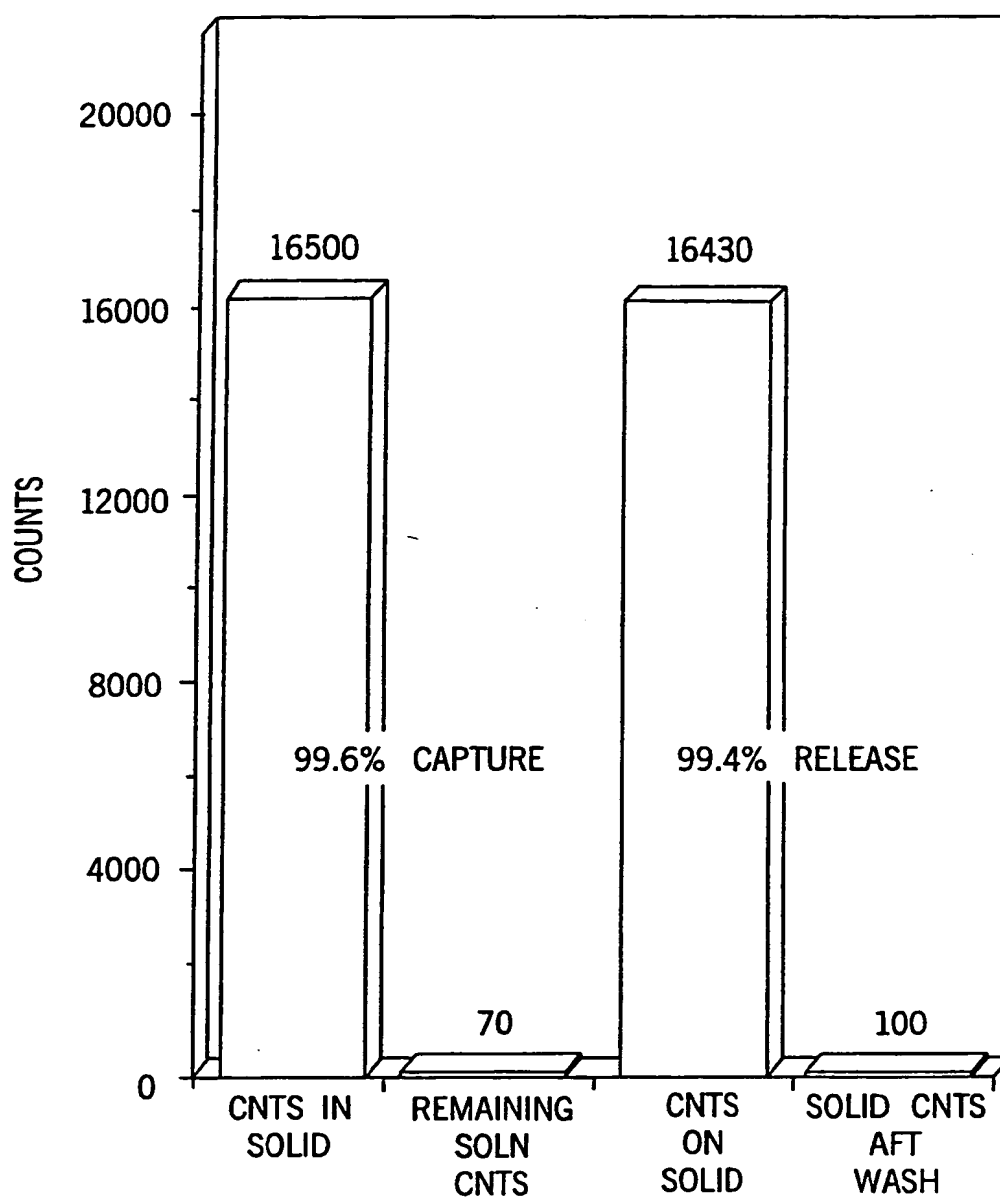
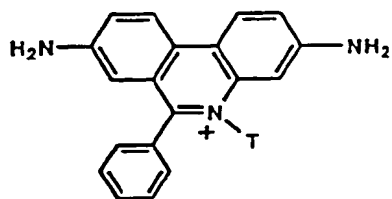


FIG. 12



phenathridinium

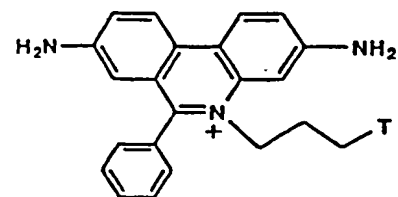
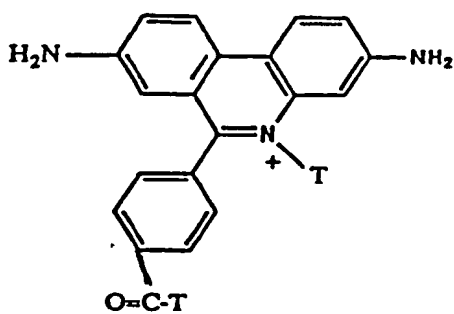
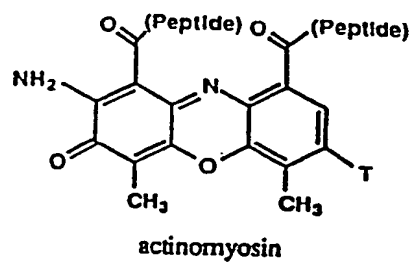
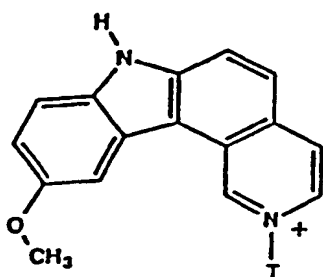
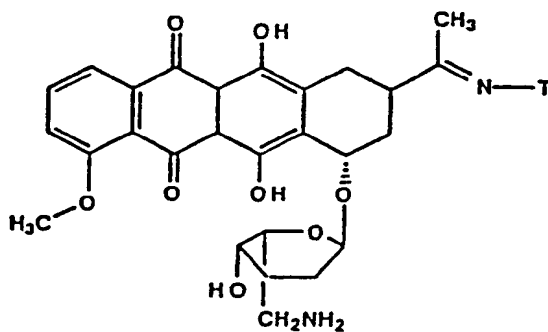
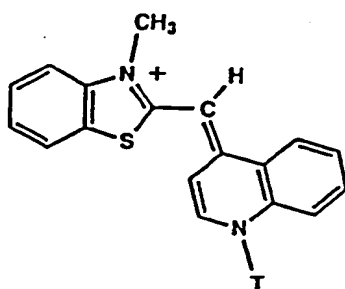
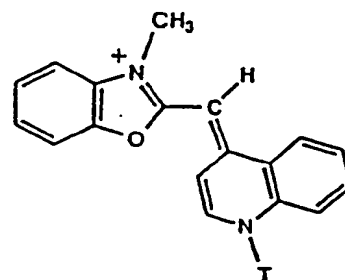
123456

FIGURE 13A

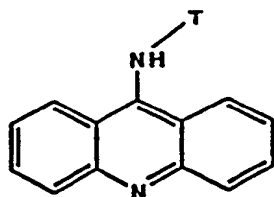
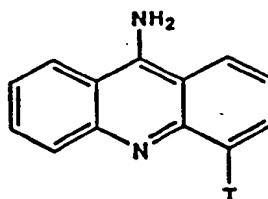
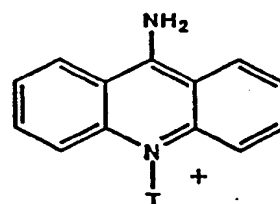
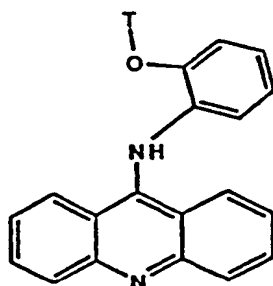
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thiazole orange

7

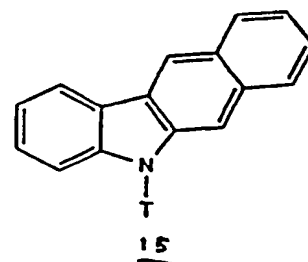
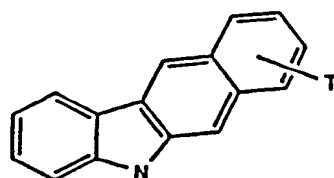
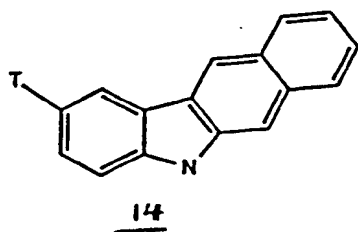
oxazole yellow

8910111213

9-aminoacridines

FIGURE 13B

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carbazoles

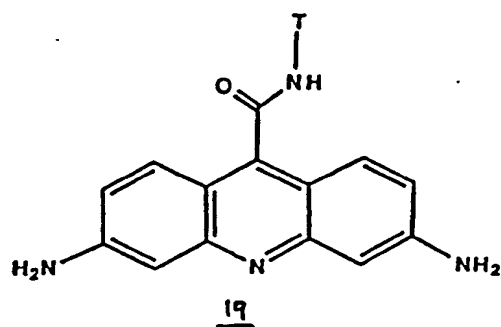
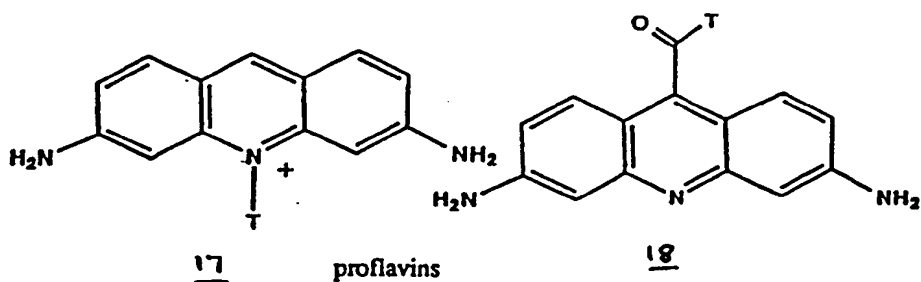
16

FIGURE 13C

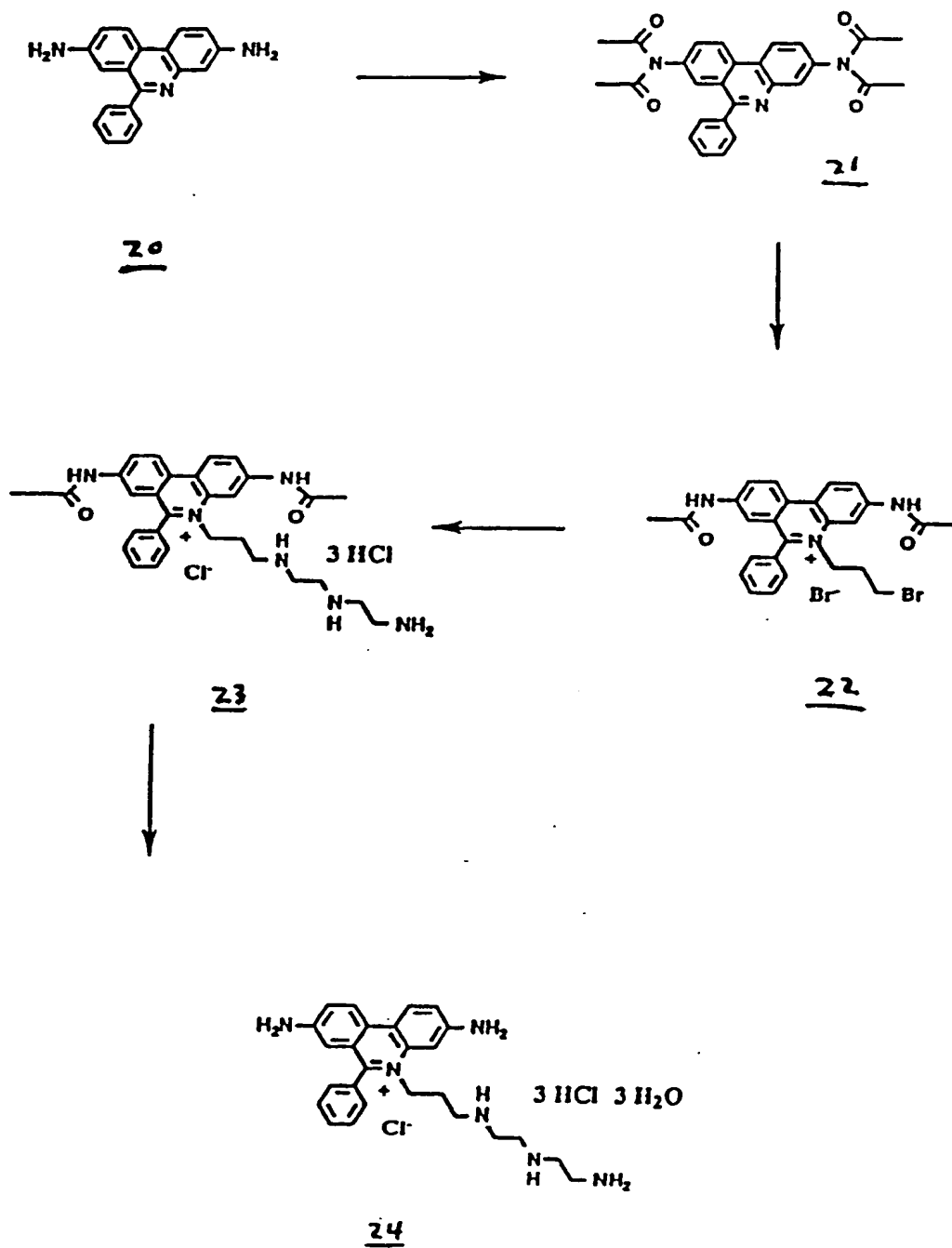


FIGURE 14

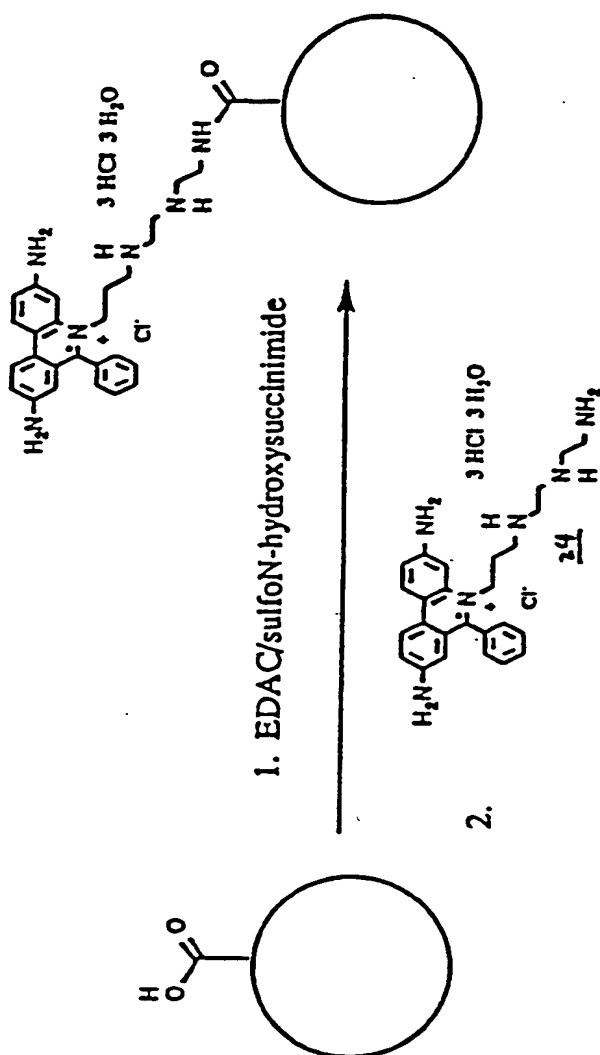


FIGURE 15

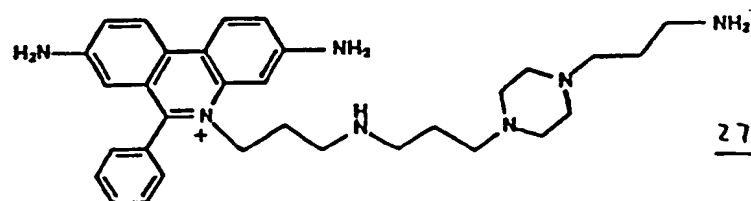
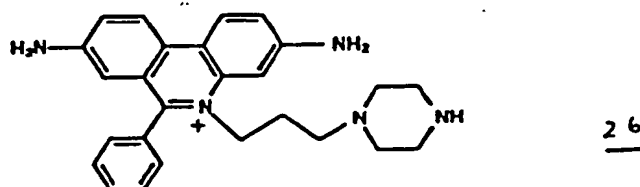
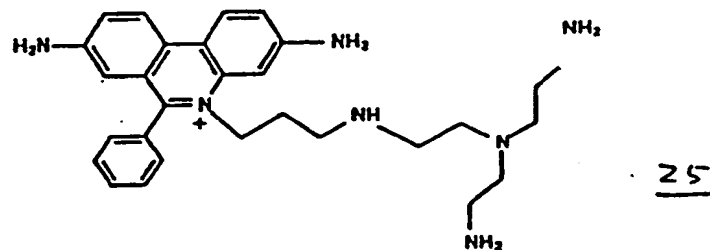


FIGURE 16

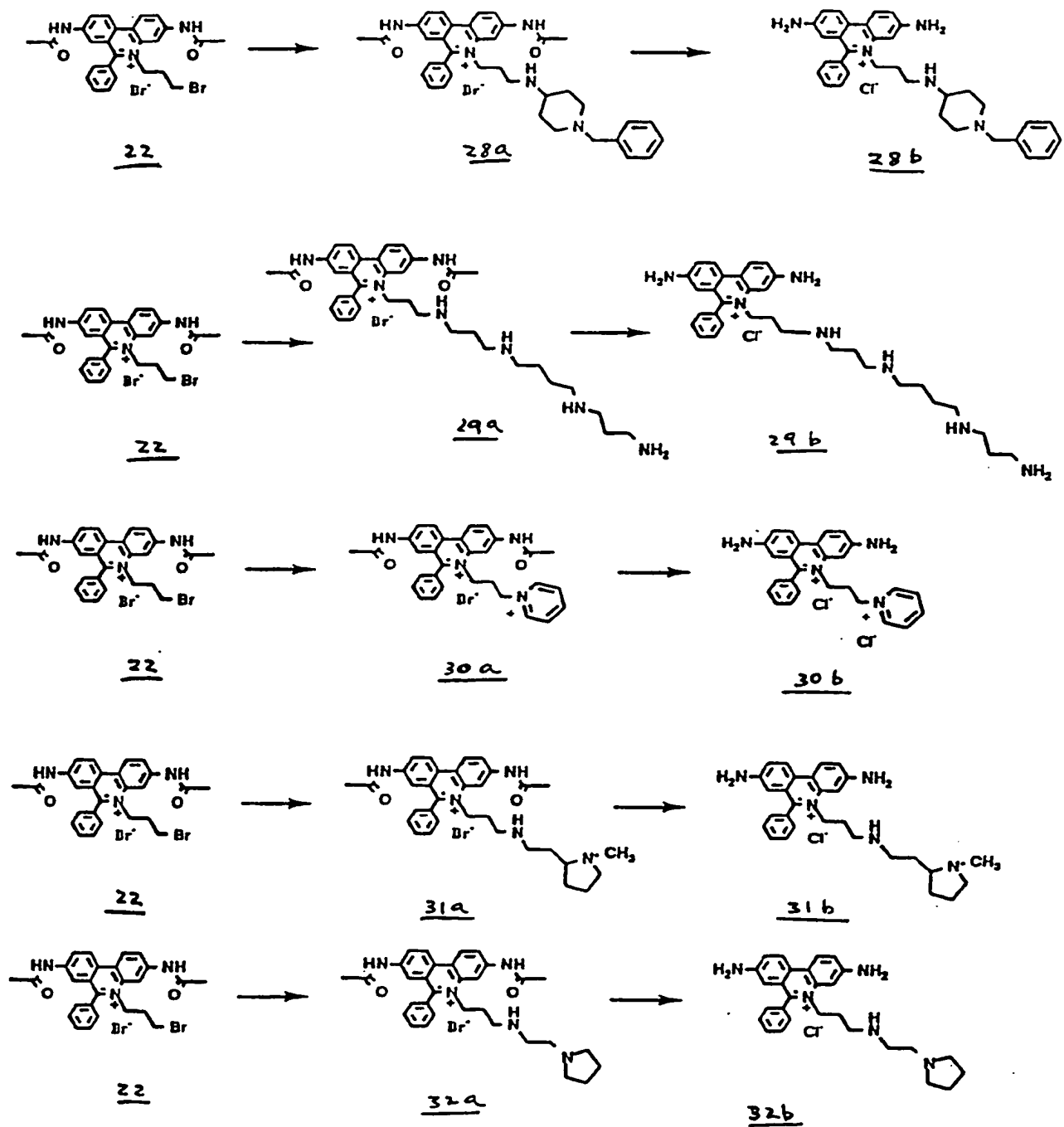


FIGURE 17A.

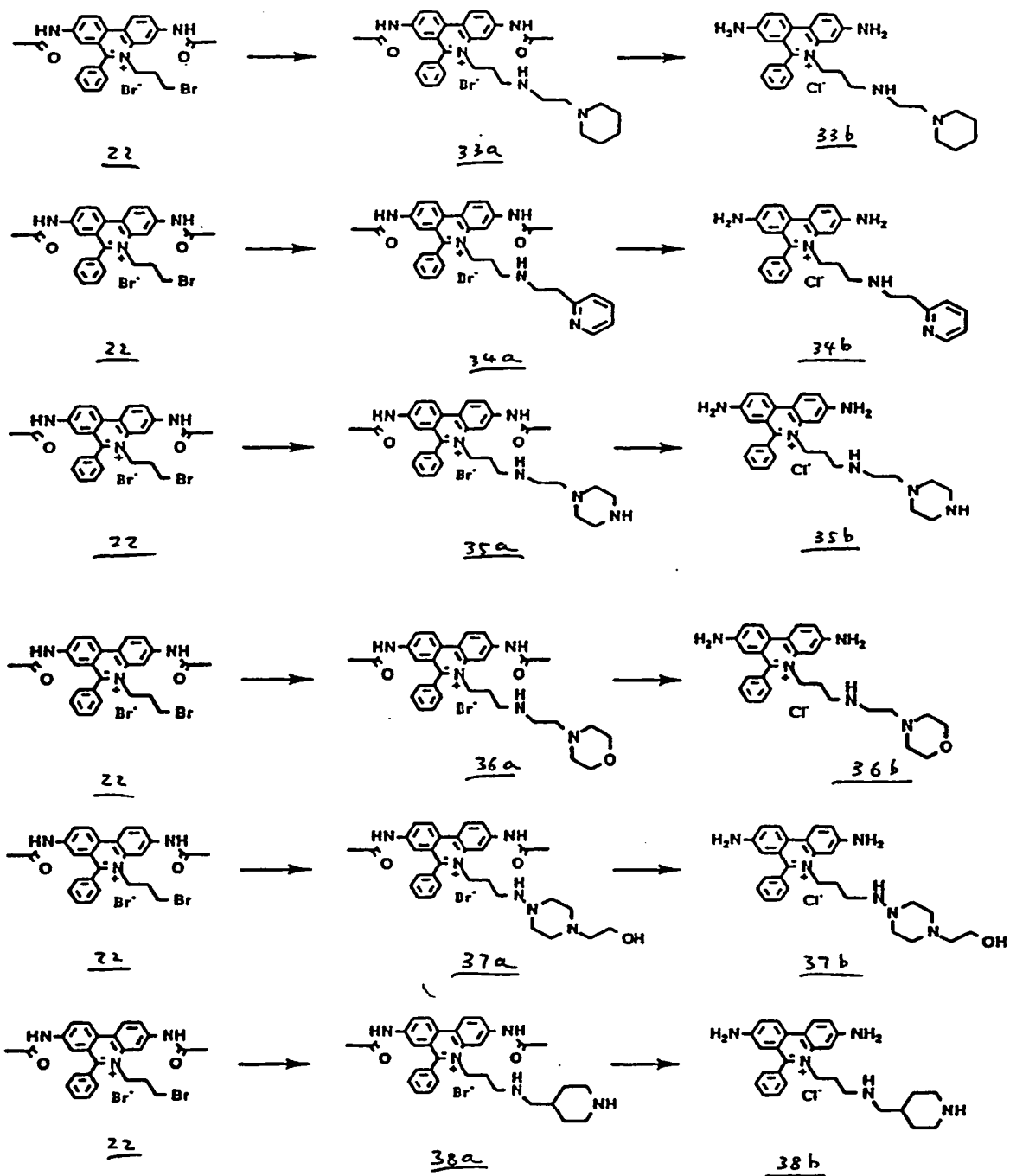


FIGURE 17B

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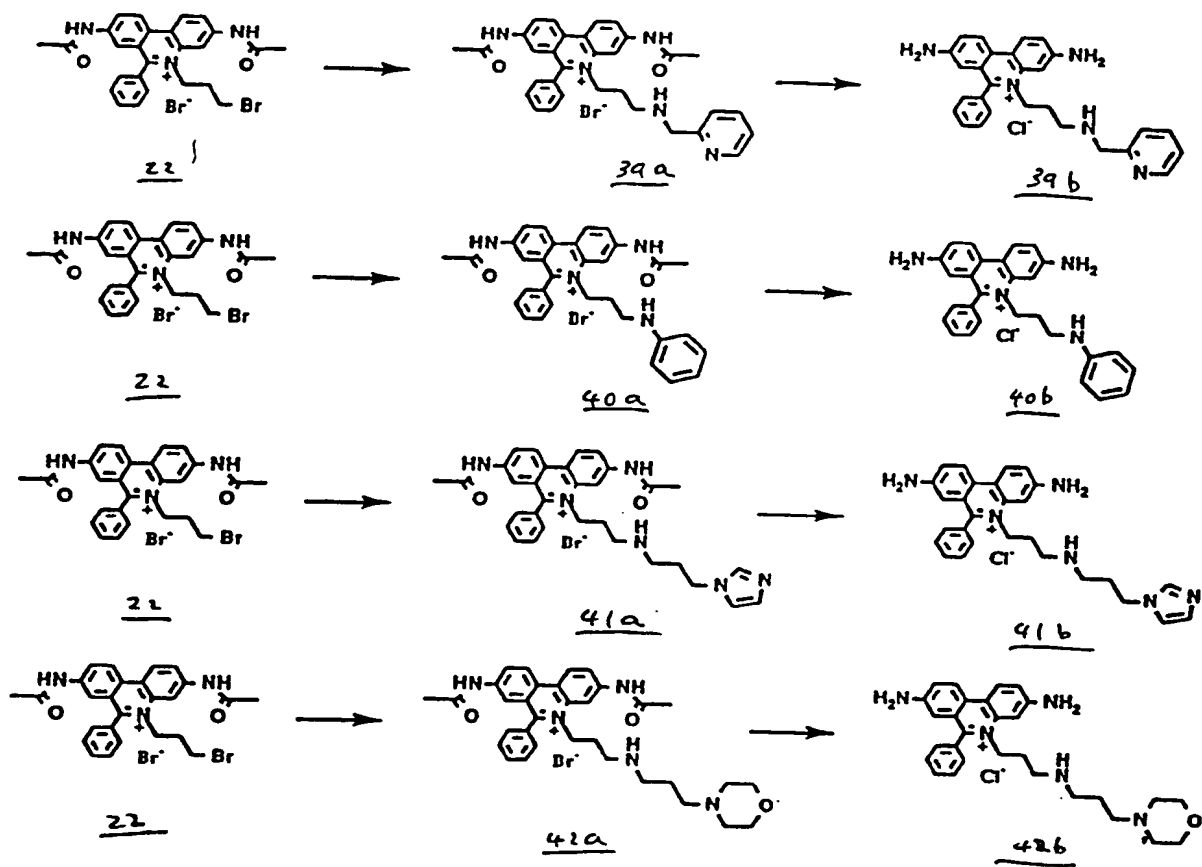


FIGURE 17C

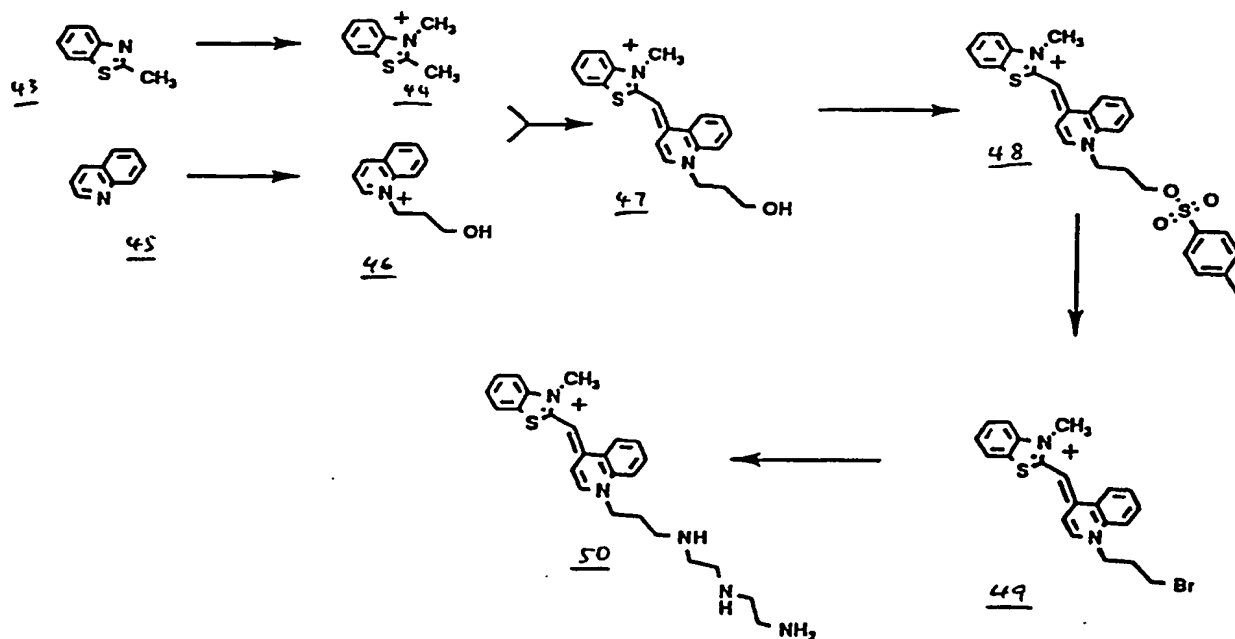


FIGURE 18

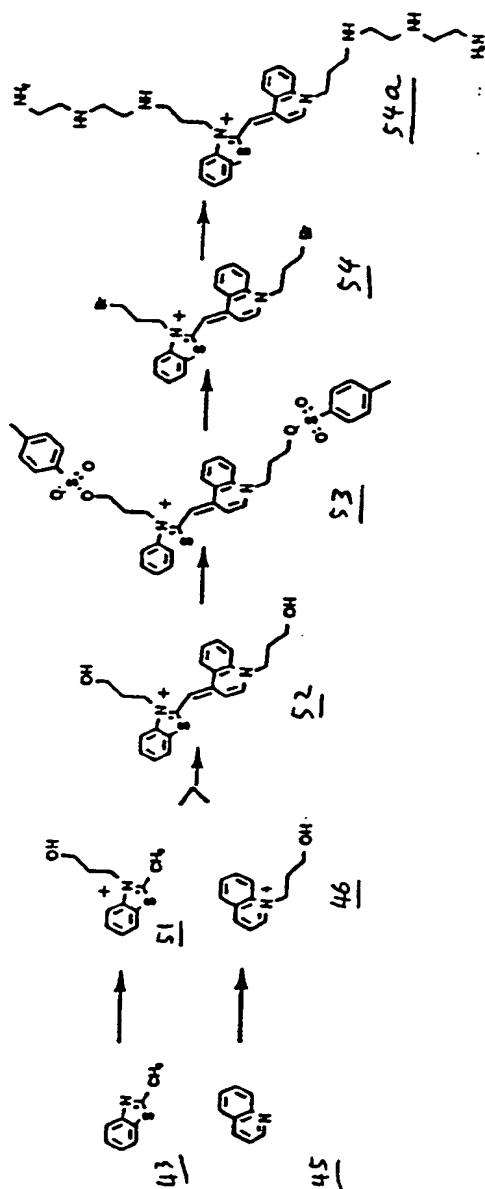
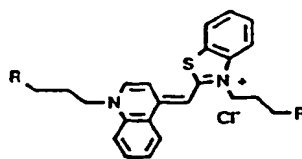


FIGURE 19



54 a - 54 p

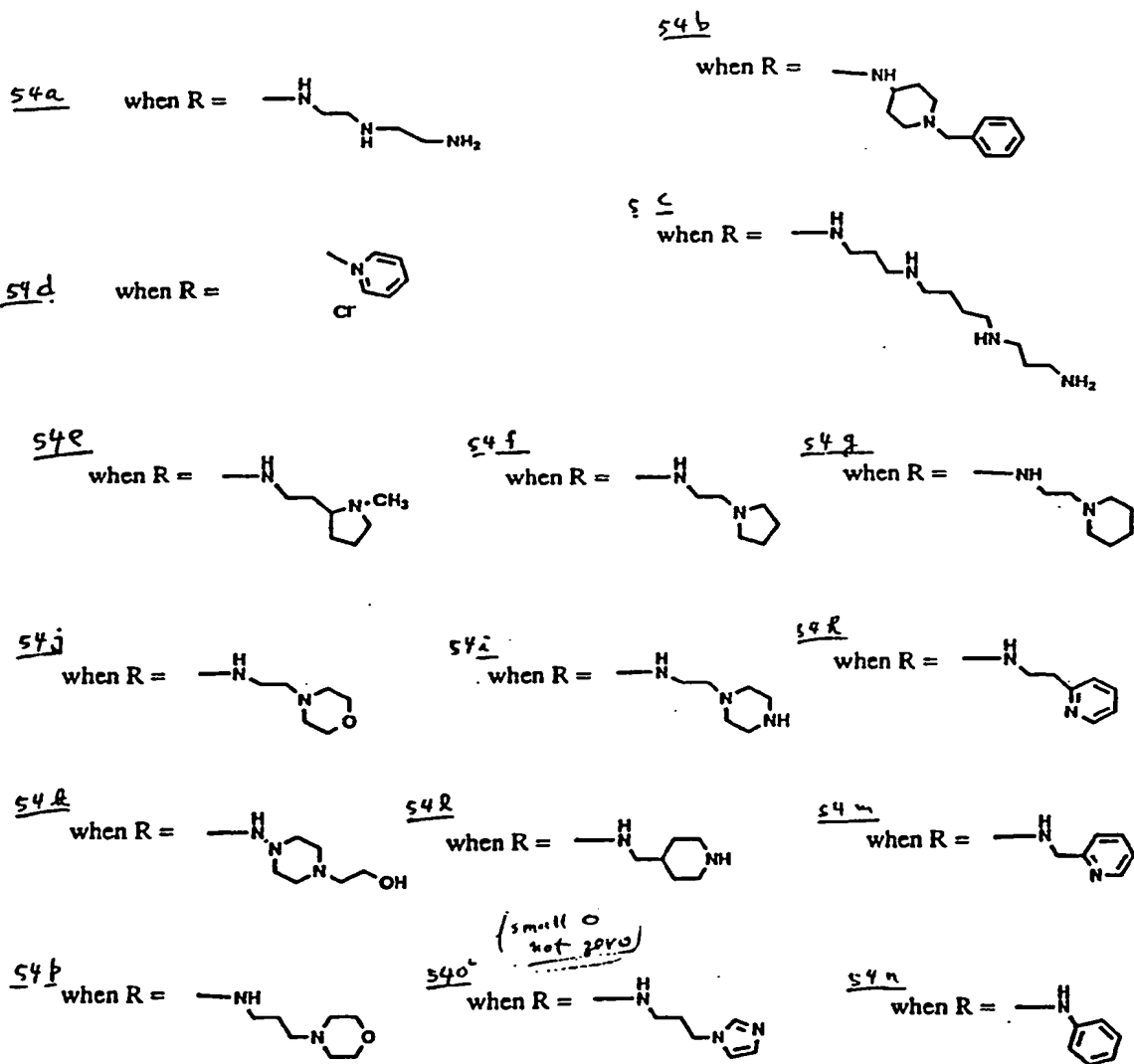


FIGURE 20

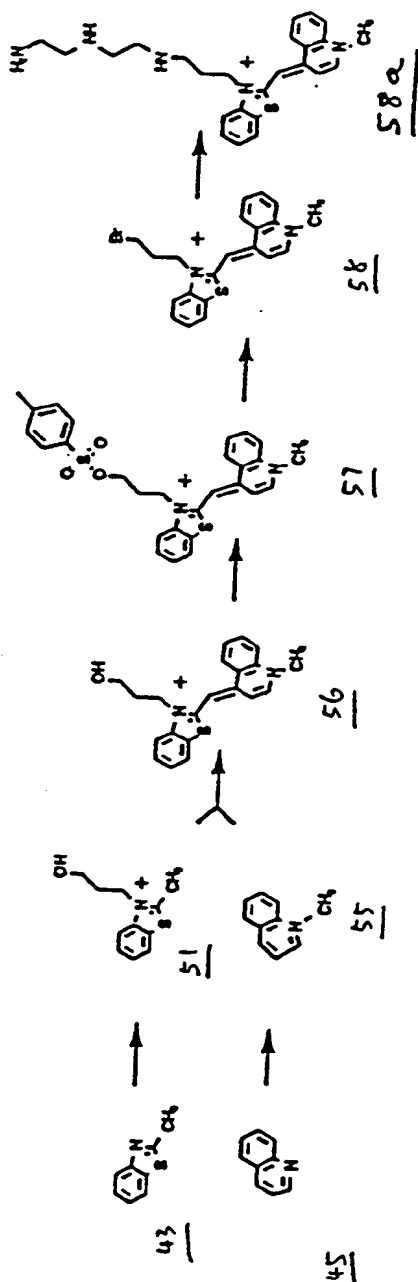


FIGURE 21

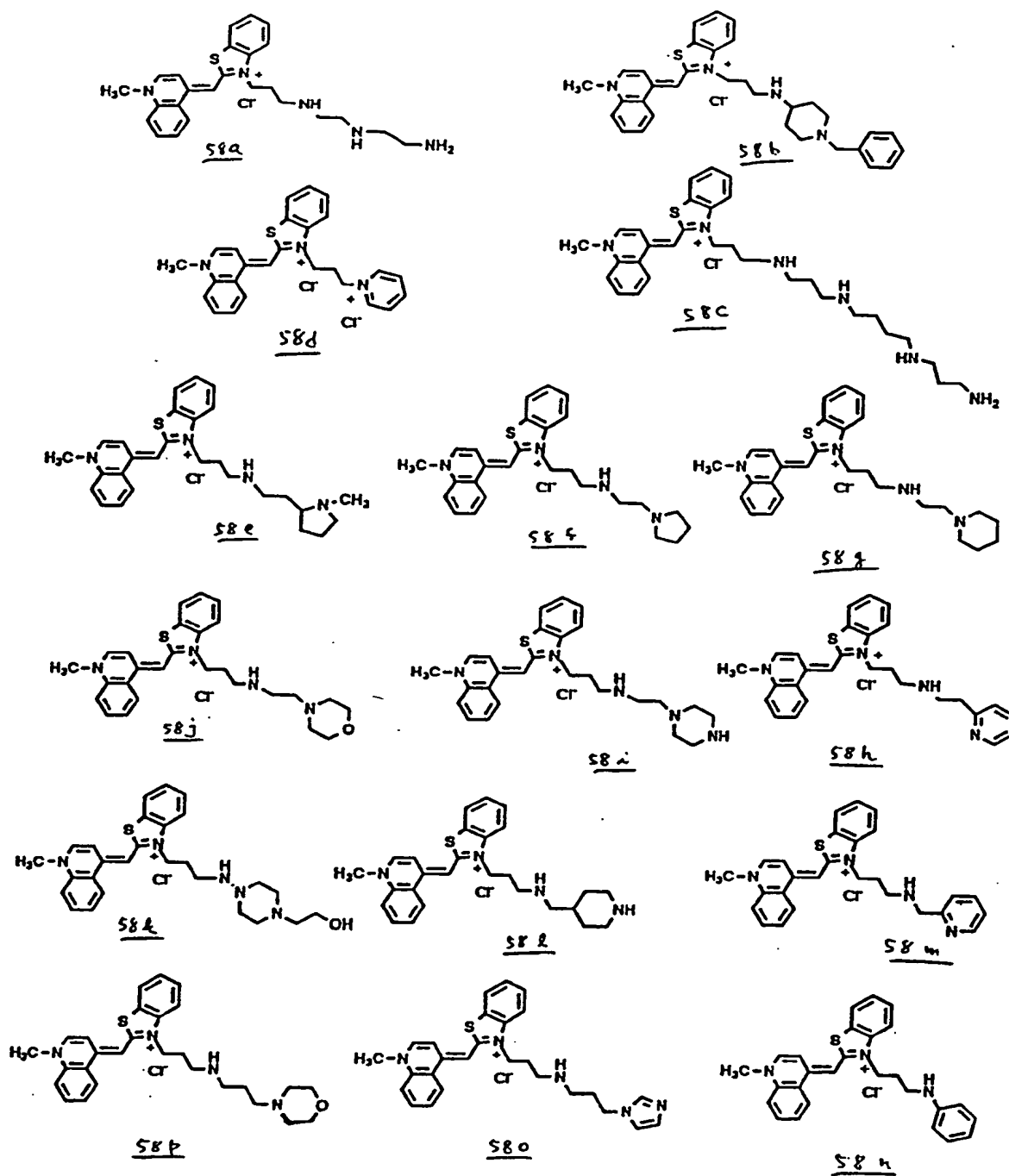


FIGURE 22

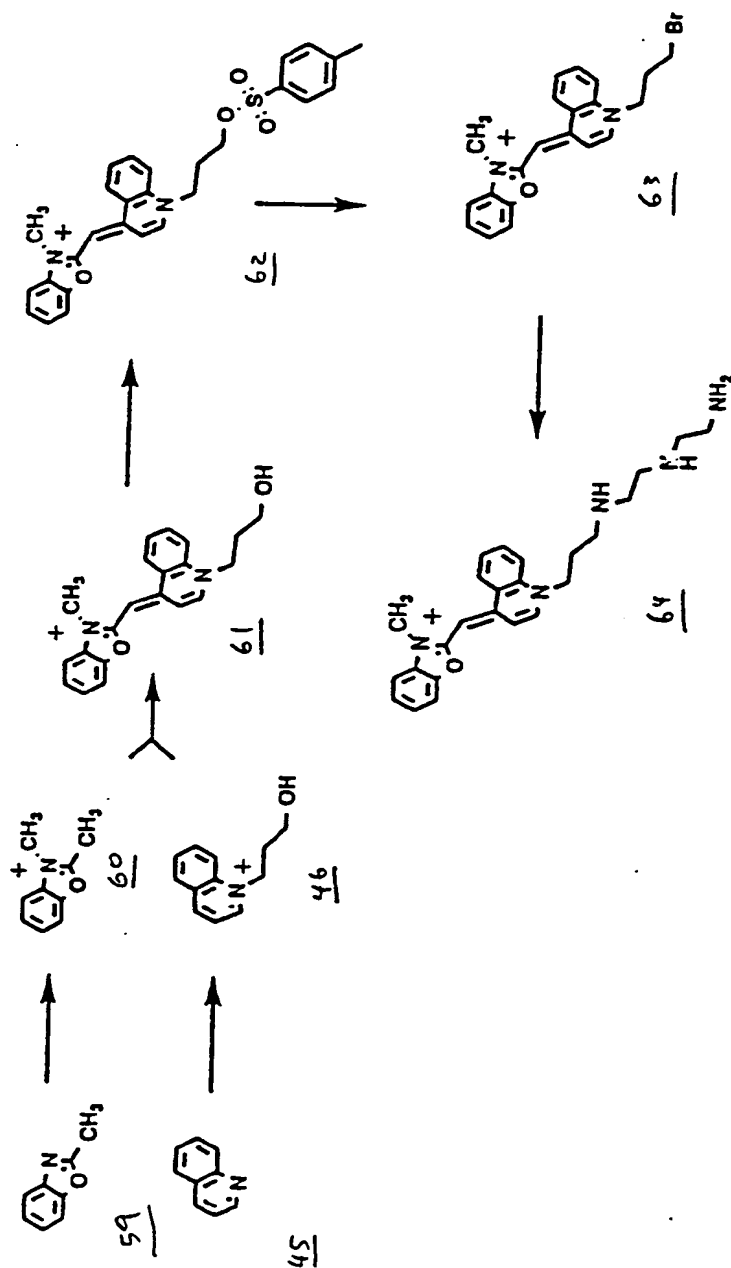


FIGURE 23

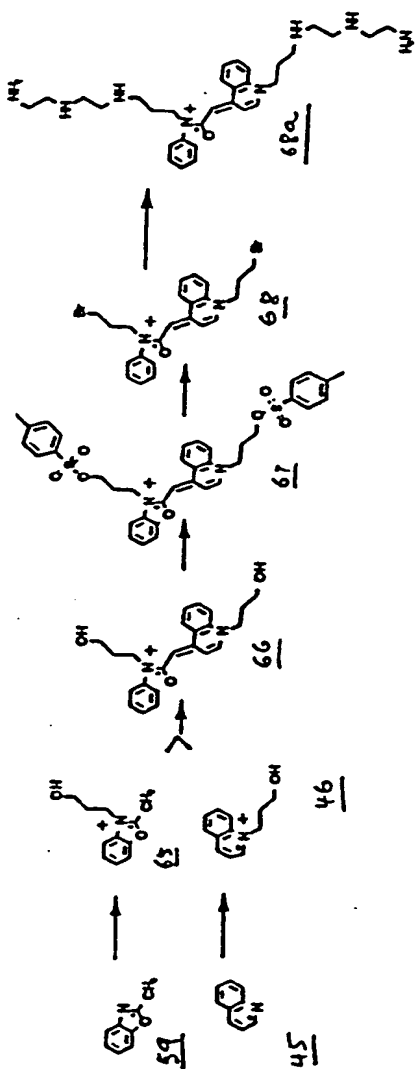


FIGURE 24

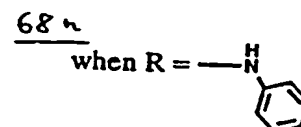
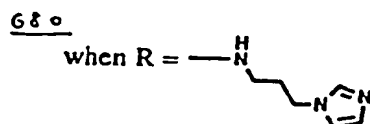
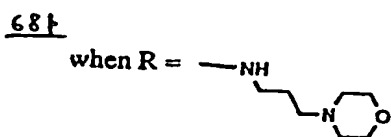
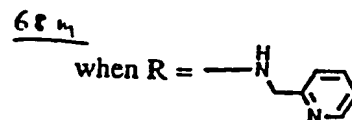
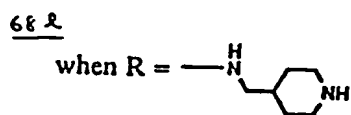
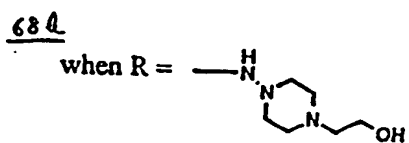
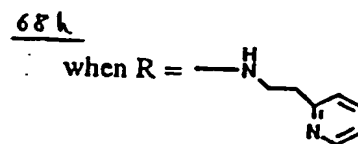
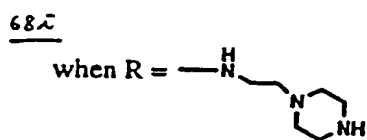
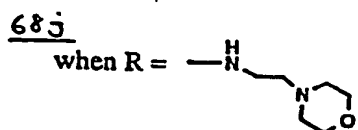
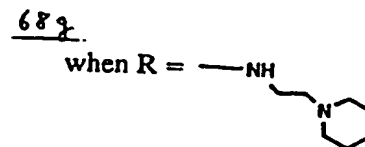
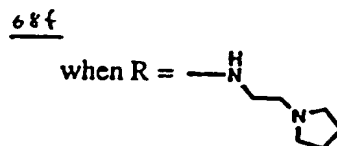
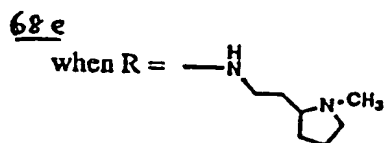
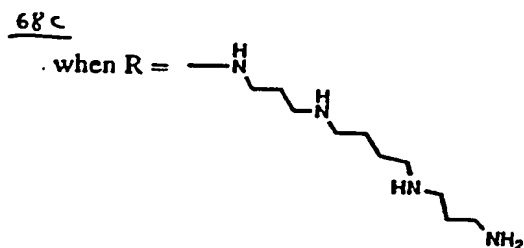
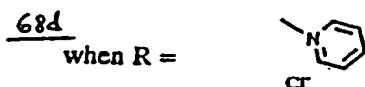
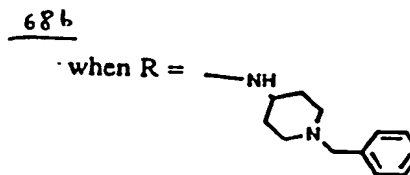
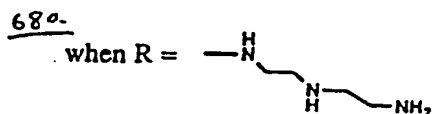
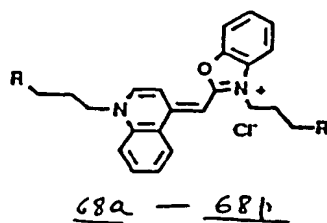


FIGURE 25
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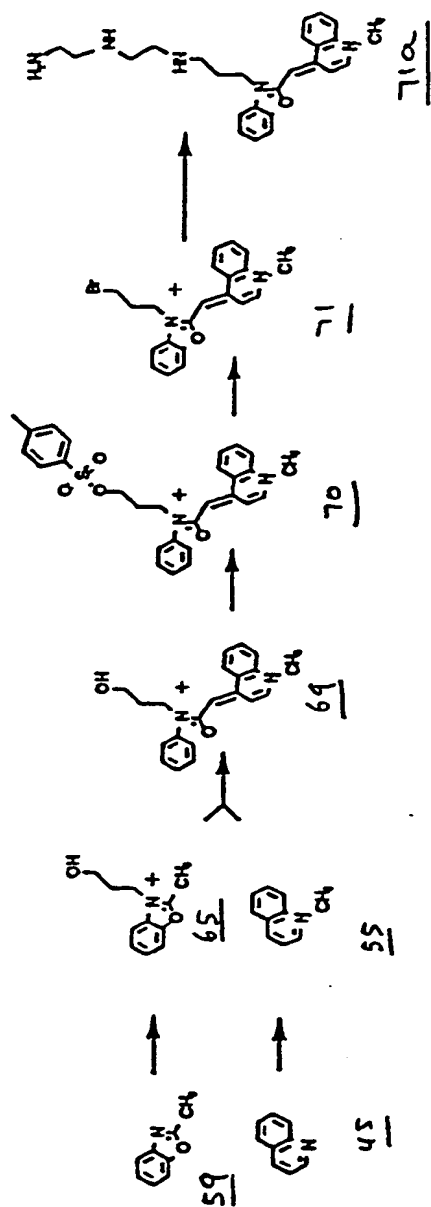


FIGURE 26

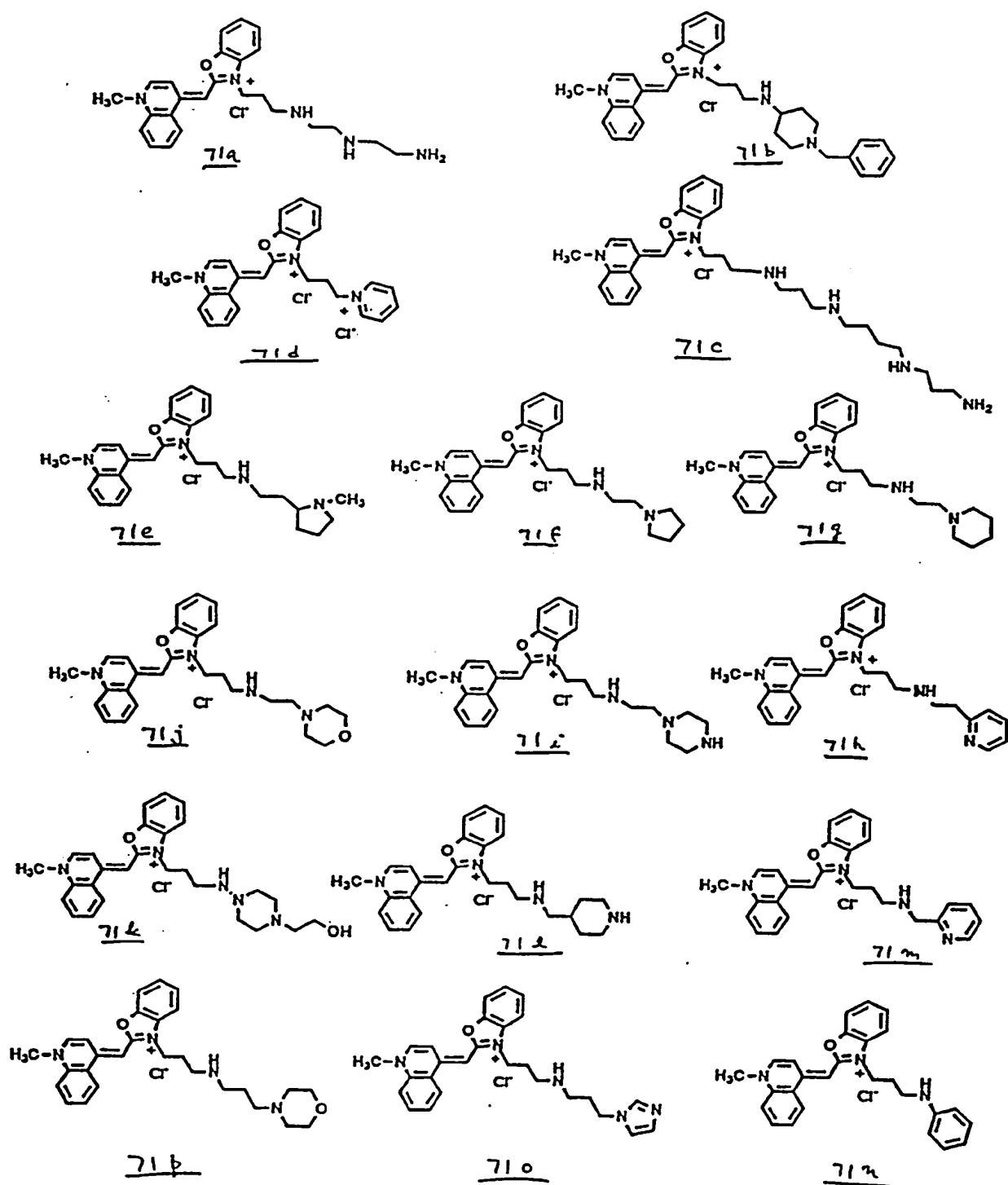


FIGURE 27

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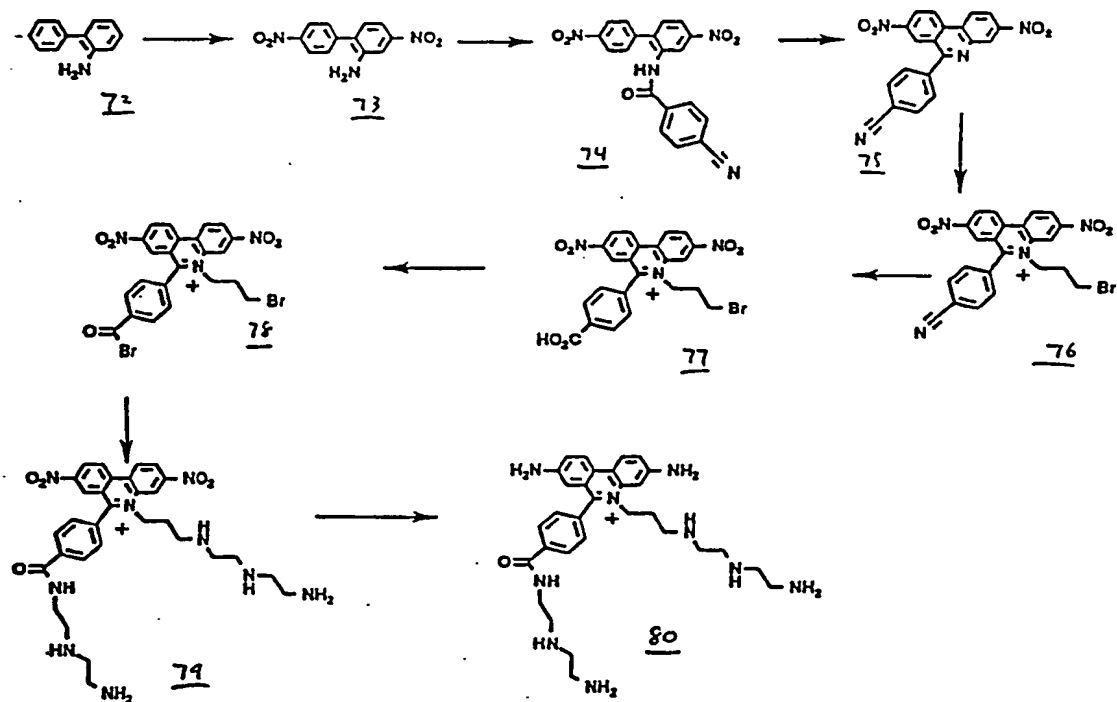


FIGURE 28

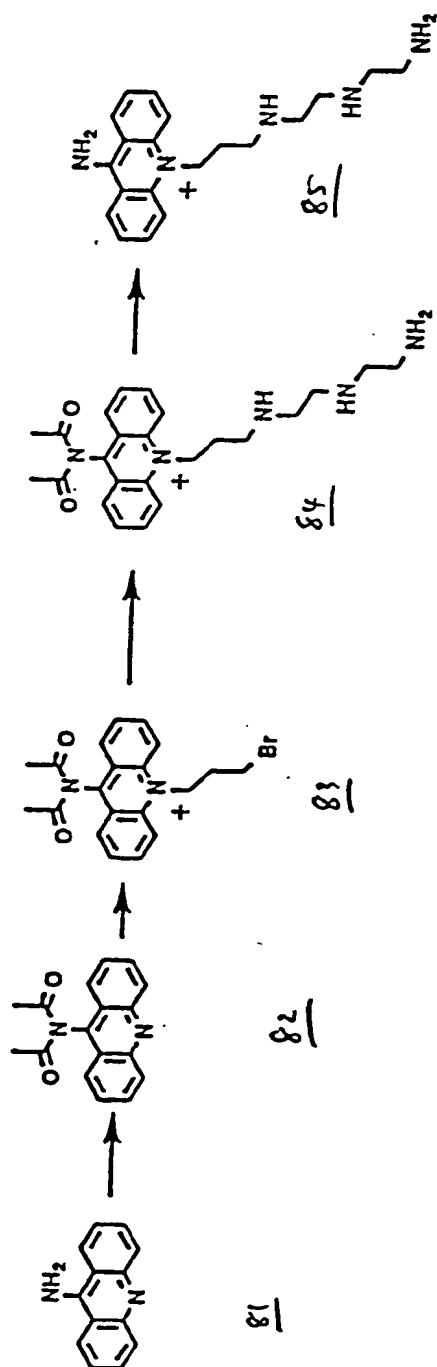


FIGURE 29

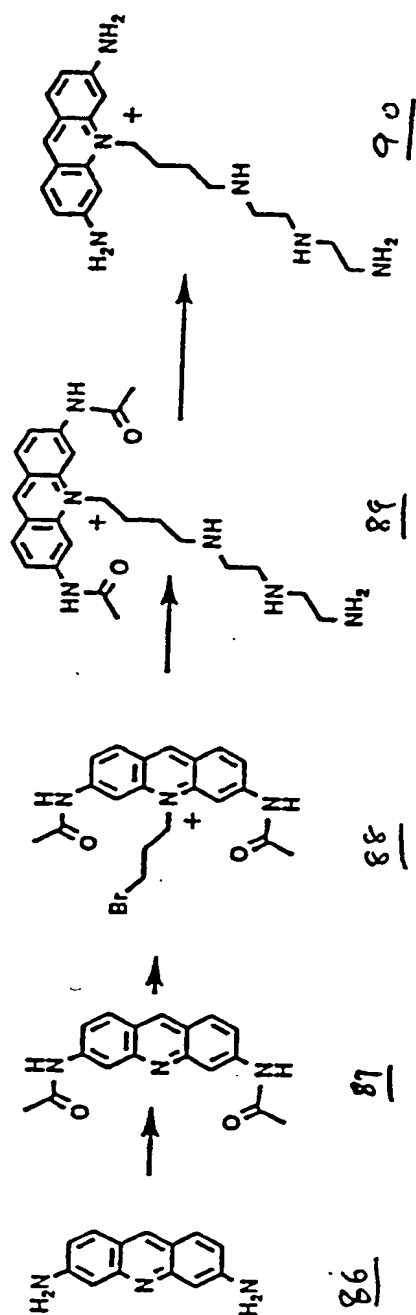


FIGURE 30

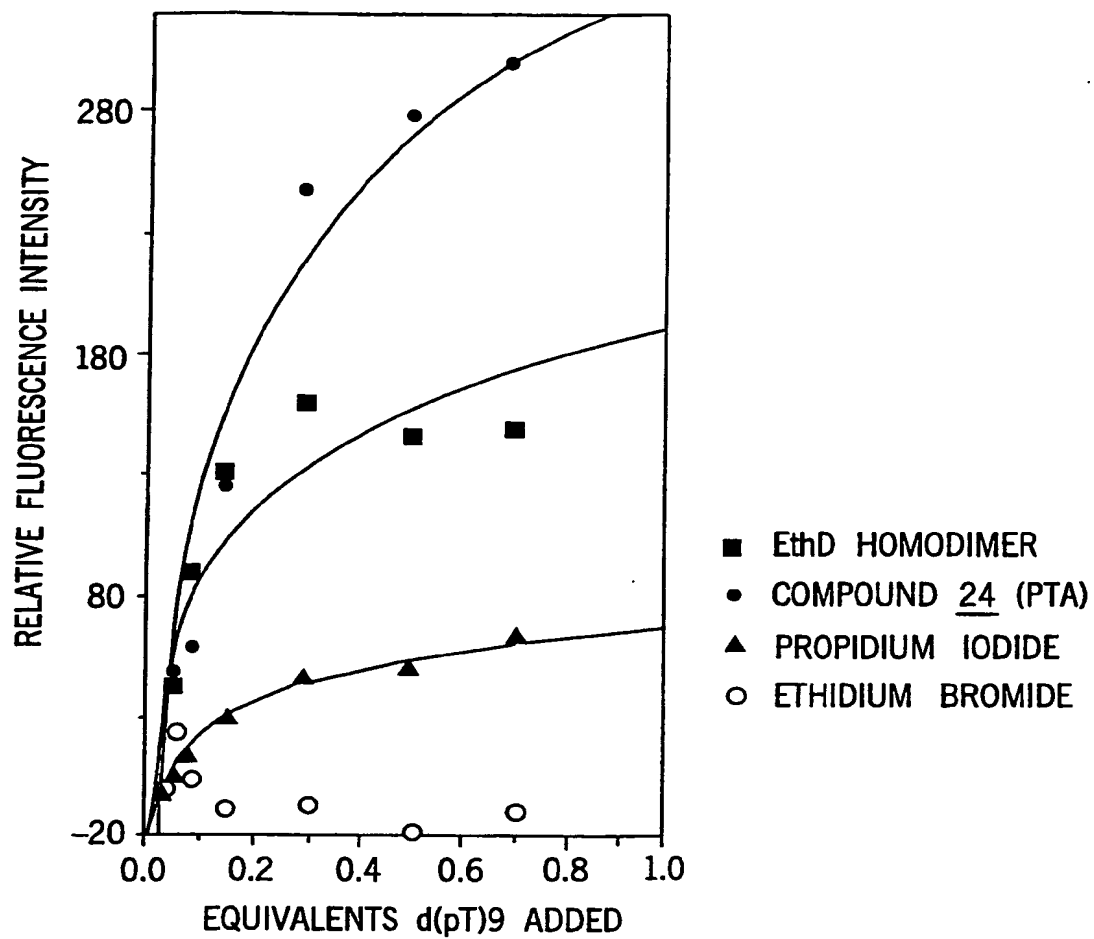


FIG. 31

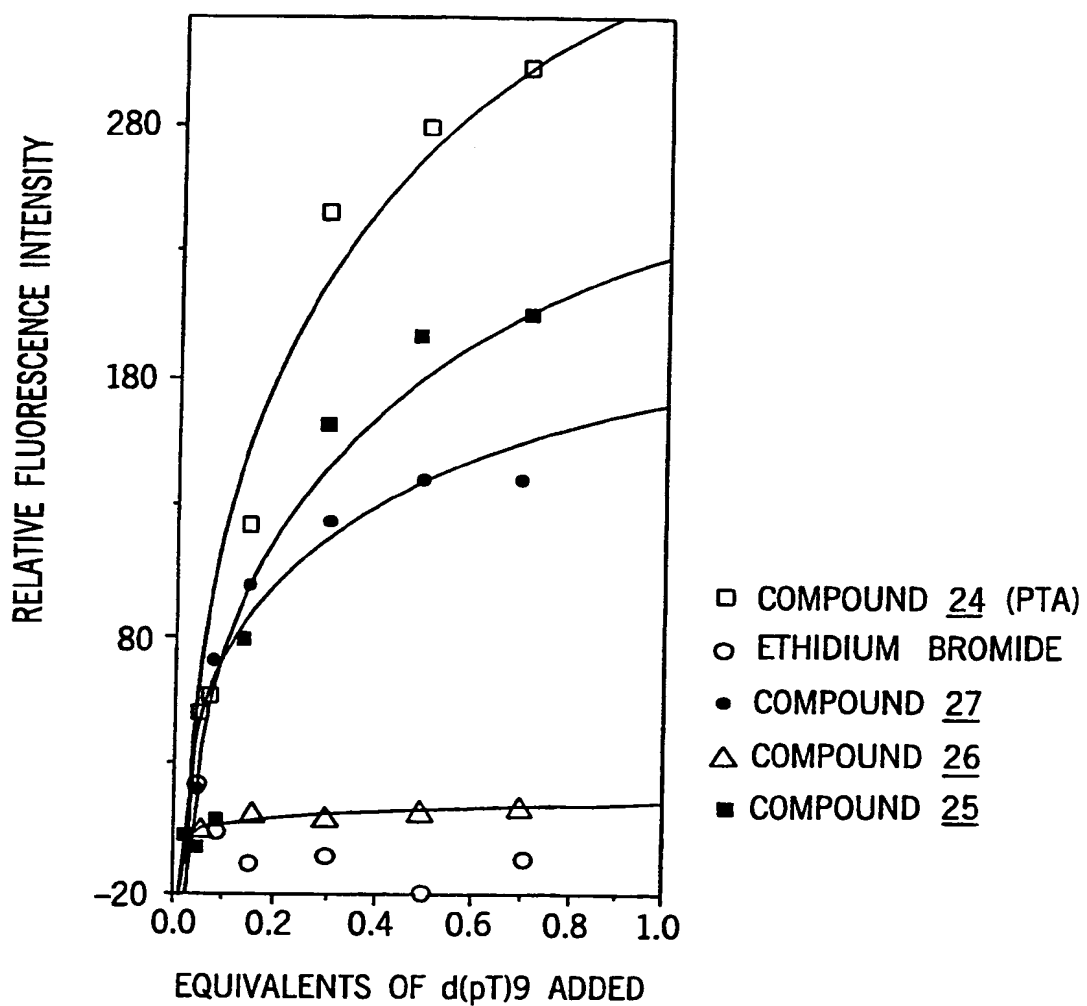


FIG. 32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/07130

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07D221/12 C07D417/06 C07D413/12 C07D219/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 08165 (ISIS INNOVATION LIMITED) 29 April 1993 see claims ---	1-30
A	NUCLEIC ACIDS RESEARCH, vol.19, no.2, 25 January 1991, ARLINGTON, VIRGINIA US pages 327 - 333 HAYS S. RYE ET AL 'High sensitivity two-color detection of double-stranded DNA with a confocal fluorescence gel scanner using ethidium homodimer and thiazole orange' see the whole document --- -/--	1-30

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

30 September 1994

Date of mailing of the international search report

13. 10. 94

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Henry, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH, vol.20, no.11, 11 June 1992, ARLINGTON, VIRGINIA US pages 2803 - 2812 HAYS S. RYE 'Stable fluorescent complexes of double-stranded DNA with bis intercalating asymmetric cyanine dyes:properties and applications' see the whole document ---	1-30
A	US,A,4 665 184 (PETER B. DERVAN ET AL) 12 May 1987 ---	
P,X	NUCLEIC ACIDS RESEARCH, vol.21, no.24, 11 December 1993, ARLINGTON, VIRGINIA US pages 5727 - 5735 SCOTT C. BENSON ET AL 'Heterodimeric DNA-binding dyes designed for energy transfer : synthesis and spectroscopic properties' see the whole document ---	1-6
P,X	NUCLEIC ACIDS RESEARCH, vol.21, no.24, 11 December 1993, ARLINGTON, VIRGINIA US pages 5720 - 5726 SCOTT C. BENSON ET AL 'Heterodimeric DNA-binding dyes designed for energy transfer : stability and applications of the DNA-complexes' see the whole document -----	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/07130

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The definition of substituents is too general and only partly supported by the examples given in the descriptive part of the application. Guided by the spirit of the application the search was carried out on the basis of the examples. (CF Art. 6 Guidelines Exam. Part B Chapt. III, 3.6, 3.7)
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US 94/07130

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9308165	29-04-93	EP-A- 0610283	17-08-94
US-A-4665184	12-05-87	US-A- 4942227	17-07-90

Form PCT/ISA/210 (patent family annex) (July 1992)

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- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

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